# Studies on Immuno-molecular Pathways Triggered by *Chlamydia trachomatis* Infection in First Trimester Spontaneous Abortion

**THESIS**

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by

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and

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**CERTIFICATE**

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# Dedication

*Dedicated to ones who gave me life and grew me up. Those, who were always supportive, I owe them each moment of my life and praise them in every breath.*

*It is with my deepest gratitude and warmest affection I Dedicate this thesis to my Parents and Husband whose continuous encouragement has helped me to reach this milestone.*

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Place: New Delhi

Date: **(NAMITA SINGH)**

## *Abstract*

*Chlamydia trachomatis* is the etiology of majority of sexually transmitted diseases. Colonization of *C. trachomatis* in the genital tract during early pregnancy has been associated with spontaneous abortion in women. Although an association between *C. trachomatis* infection and spontaneous abortion is acknowledged in various studies, there is a paucity of mechanistic studies till date on the underlying immunomolecular pathway leading to spontaneous abortion in infected women. Hence the cyclooxygenase derived prostaglandin-signalling pathway along with proinflammatory cytokines, hormones, matrix metalloproteinases and their inhibitors was studied in *C. trachomatis*-positive spontaneous aborters (SA) for an improved understanding of varied immunomolecular aspects during spontaneous abortion in women infected with *C. trachomatis.*

In the present study, firstly, the molecular detection of *C. trachomatis* was performed in the Endometrial Currettage Tissues (ECT) of aborters by using PCR and real time PCR assays. The present study confirmed that *C. trachomatis* infection should be taken into consideration as far as pregnancy failures are concerned. The expression of COX-1 and COX-2 genes was further studied in *C. trachomatis*-positive SA in comparison to *C. trachomatis*-negative SA and uninfected controls. The expression pattern of COX-1/ COX-2 was also compared between recurrent SA (RSA) in and sporadic SA (SSA). Furthermore, the prostaglandin E/ F/ I receptors (EP-1, EP-2, EP-3, EP-4, FP and IP) were also quantified in the ECT of aborters. Mean serum estrogen and progesterone concentration was also measured in *C. trachomatis*-positive SA, *C.*

*trachomatis*-negative SA and controls. Our findings delineated that increased expression of COX-2 and PG receptors particularly the contractile receptors and hormonal imbalance could be a possible risk factor for abortion in *C. trachomatis*-infected SA.

In addition, an inflammatory response was seen in *C. trachomatis*-infected SA. This was deduced on the basis of significantly increased cytokines (TNF-α, IFN-γ, IL-8, TGF-β1, TGF-β2) in the ECT of *C. trachomatis*-positive SA and a positive correlation was found between TNF-α/ TGF-β1/ TGF-β2 and COX-2 in *C. trachomatis*-infected aborters. In the study, it was suggested that an aberration in cytokine production also constitutes an important contributing factor in *C. trachomatis*-positive SA and data support the hypothesis of Th1 cytokine involvement in the immunopathogenesis of SA. The study further revealed the involvement of matrix metalloproteinases (MMP-2/ MMP-9) and their inhibitors (TIMP-1/ TIMP-3) in endometrial extracellular matrix (ECM) turnover in *C. trachomatis*-infected SA. Expression of MMP-2/ MMP-9/ TIMP-1/ TIMP-3 was estimated in the ECT of *C. trachomatis*-positive SA in comparison to the *C. trachomatis*-negative SA and controls and it was concluded that dysregulated MMPs/ TIMPs could degrade the endometrial ECM leading to spontaneous abortion in *C. trachomatis*-infected women.

Conclusively these results inferred that *Chlamydia trachomatis* infection leads to spontaneous abortion in women by triggering the cytokines and further the COX-derived PG signally pathway and MMPs.

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## *Abbreviations*

| | |
|---|---|
| **COX** | Cyclooxygenase |
| **EB** | Elementary bodies |
| **ECM** | Extra cellular matrix |
| **ECT** | Endometrial curettage tissue |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **GA** | Gestational age |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **IFN-γ** | Interferon-gamma |
| **IL-8** | Interleukin- 8 |
| **IRSM** | Idiopathic recurrent spontaneous miscarriage |
| **LPS** | Lipopolysaccharide |
| **MMP** | Matrix metalloproteinases |
| **MOMP** | Major outer membrane protein |
| **NAAT** | Nucleic acid amplification test |
| **OD** | Optical density |
| **PGs** | Prostaglandins |
| ***q*-PCR** | Quantitative real time PCR |
| **RB** | Reticulate bodies |
| **RSA** | Recurrent spontaneous aborters |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **SA** | Spontaneous aborters |
| **SSA** | Sporadic spontaneous aborters |
| **TAE** | Tris-acetate-EDTA |
| **TIMP** | Tissue Inhibitors of matrix metalloproteinases |

# CHAPTER 1

# INTRODUCTION, REVIEW OF LITERATURE, AIMS & OBJECTIVES

## 1.1. Introduction

Spontaneous abortion is pregnancy loss before 20 weeks of gestation from the last menstrual period and less than 500 g of fetal weight **(WHO, 2001)**. It can be further divided in to two categories on the basis of number of abortions: Sporadic spontaneous abortion; only one abortion and recurrent spontaneous abortion (referred to as *habitual abortion*) historically defined as 3 or more consecutive pregnancy losses prior to 20 weeks of gestation from last menstrual period **(Stephenson *et al.,* 1996)**. It is a frequent outcome in pregnancy and is one of the most difficult areas in reproductive medicine, since the etiology is often unknown. It affects approximately 1-5% of all women of childbearing age, whereas approximately 15% of all clinically recognized pregnancies result in spontaneous abortion. In addition, there are many more pregnancies that fail prior to being clinically recognized. Only 30% of all conceptions result in a live birth (**Macklon *et al*., 2002)**. The incidence of recurrent spontaneous abortion is approximately 1 in 300 pregnancies. Studies have identified various factors including genetic, endocrine, infectious, anatomical and autoimmune in approximately 50% of patients with recurrent spontaneous abortions, while the mechanisms in the other 50% remain unexplained **(Ford *et al.,* 2009).**

Many sexually transmitted pathogens and their infections are a recognized cause of fetal loss in animals and humans (**Deruaz & Luster, 2015).** *Chlamydia trachomatis* infection is a common cause of sexually transmitted diseases in USA, with approximately 1.3 million infections reported to Centres for Disease Control in 2014 **(CDC 2014)**. The incidence and prevalence of *C. trachomatis* in South-East Asia region was reported as 7.2 million and 8 million, respectively by World Health Organization **(WHO 2015).**

Furthermore, *C. trachomatis* has also been found associated with adverse obstetric outcome including spontaneous abortion **(Rastogi *et al.,* 2000; Baud *et al.,* 2011)**. A handful of initial studies attempted to establish a relationship between serological evidence of past *C. trachomatis* infection and spontaneous abortion **(Ouinn *et al.*, 1997; Wilkowska-Trojniel *et al.,* 2009)**. These investigations were conducted among women who had experienced recurrent pregnancy loss or who had an unsuccessful implantation during *in vitro* fertilization **(Osser *et al.,* 1996)**. Recent studies have further established an association between *C. trachomatis* infection and spontaneous abortion in women **(Baud *et al.,* 2008/ 2011; Jahromi *et al.,* 2010; Ahmadi *et al.,* 2016).**

Despite a plethora of literature describing an association between *C. trachomatis* infection and spontaneous abortion, there is a paucity of mechanistic studies till date on the underlying immunomolecular pathway leading to spontaneous abortion in infected women. The bacterial cell wall component, lipopolysaccharide (LPS) induces a marked inflammatory response that is mediated primarily by the activation of macrophages and the release of cytokines and further prostaglandins (PG) through induction of enzymes of the prostaglandin synthetic pathway, particularly Cyclooxygenase-2 (COX-2). Spontaneous abortion may result from direct stimulation of the uterine activity by chlamydial infection. Decidual and foetal membranes, especially amnion, contain large amounts of arachidonic acid- the precursor of PG synthesis **(Fig. 1.1).**

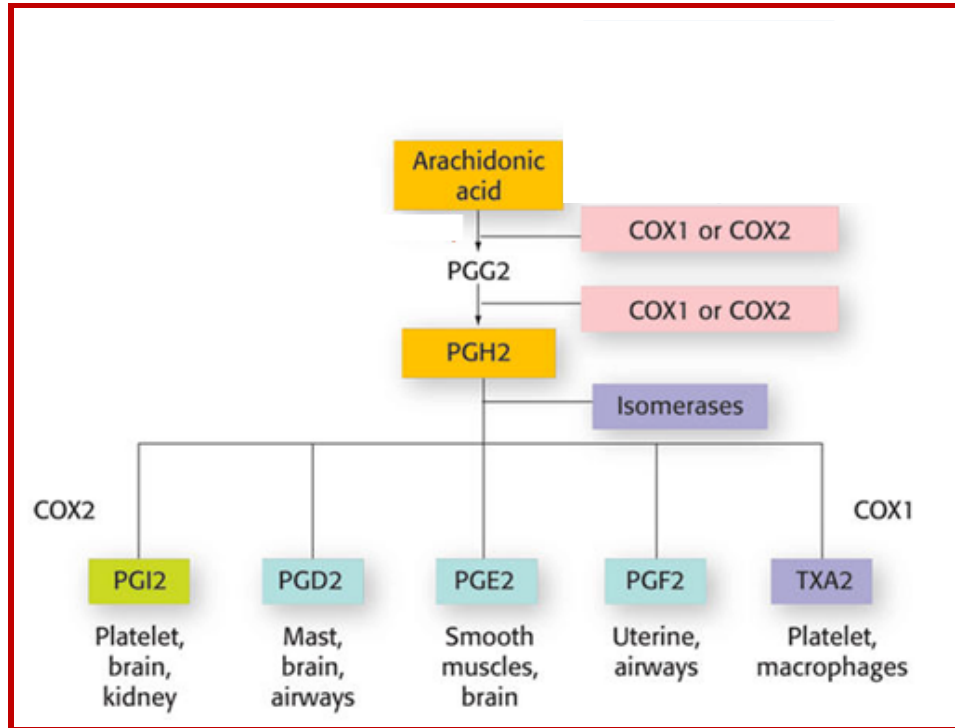



**Fig. 1.1:** Schematic representation of cyclooxygenase/prostaglandin pathway (*Challis et al.,* 2000)

In view of the above, it is evident that the molecular changes underlying the complex transition from uterine quiescence to labour are not completely studied. COX-2 is highly inducible during inflammation or in response to LPS. Recent studies demonstrated that there was a low level of COX-2 expression in trophodermal villi of patients with spontaneous abortion compared with normal pregnant women **(Yu *et al.,* 2010)**, thereby indicating that the reduced expression of COX-2 might be one of the main factors that interferes with embryo implantation ultimately resulting in abortion. Furthermore an animal model study showed that COX-2 was increased upto six-fold in decidua of mice treated with bacterial LPS **(Silver *et al.,* 1995)**. Another report demonstrated significant elevation in expression of COX-2 mRNA in endometrium

obtained from women with heavy menstrual bleeding and suggested that COX-2 led to increased PGs production and signalling (**Smith *et al.,* 2007**).

COX-2 mediates the conversion of arachidonic acid into $PGH_2$, which is then converted into various PGs such as $PGF_{2\alpha}$, $PGD_2$, $PGE_2$ and $PGI_2$ by specific enzymes. Uteroplacental PGs are lipid mediators that play pivotal role in reproduction and maintenance and/ or termination of pregnancy **(Ni *et al*., 2002).** COX-2 is important in the regulation of PG formation. Since PGs mediate the signs and symptoms of gram-negative septic shock, stimulate myometrium and are capable abortifacients, they are likely to be important mediators of LPS-induced pregnancy loss **(Hertelendy & Zakar, 2004)**. Changes in the expression of PG receptors could be involved in the maintenance of uterine quiescence for the majority of gestation and possibly, activate the uterus to contract at the time of parturition for expulsion of the foetus **(Smith *et al.,* 2001)**. LPS-induced abortion has also been found associated with PGs **(Skarnes & Harper, 1972).** The latter exert their effect through G protein-coupled receptors, designated EP, FP, DP and IP respectively **(Narumiya *et al*., 1999).** When these receptors are bound by their appropriate prostanoid ligand, they activate contractile (EP-1, EP-3, FP)/ relaxatory (EP-2, EP-4, IP) receptors. It has been suggested that changes in the expression of PG receptors throughout gestation and with labour may participate in maintenance of uterine quiescence for the majority of gestation and the switch to contractions at delivery for expulsion of the foetus **(Coleman *et al*., 1994).** Previous studies of PG receptor gene expression are limited to nonprimate species or lower segment biopsies from pregnant women. COX-2/ PGES pathway is responsible for the endometrial production of PGE2 in the bovine endometrium during estrous cycle **(Arosh *et al.,* 2002).**

Recent attention has also focussed on immunologic cytokines as potential mediators of reproductive function and their involvement in normal and abnormal pregnancy. A balanced cytokine response is thought to be important in maintaining pregnancy **(Van Bodegom *et al.,* 2007).** By infections, an inflammatory activation of endometrial immunocytes can cause excessive maternal immune response towards trophoblastic invasion and induce an early pregnancy failure in some recurrent aborters. It has been reported that many miscarriages are caused by abnormal levels of cytokines to control the inflammatory process in the body. Prolonged exposure to proinflammatory cytokines can be severely detrimental to the pregnancy and result in a loss of the fetus. Using an *ex-vivo* model, it was demonstrated that *in vitro* stimulation with LPS and *Streptococcus agalactiae* induced a differential response in IL-1β and TNF-α secretion by the amnion and choriodecidual tissues **(Zaga *et al.,* 2004)**. An altered expression of proinflammatory, anti-inflammatory, and angiogenic cytokines was found to be one of the key factors in women with idiopathic recurrent spontaneous miscarriage (IRSM) **(Banerjee *et al.,* 2013)**. LPS may affect PG production indirectly by stimulating release of inflammatory cytokines such as TNF-α that subsequently induce COX-2. *In vitro* studies provide evidence that in cells infected with chlamydiae, TNF-α induces the production of PGE2 much more effectively than in uninfected cells **(Holtmann *et al.,* 1990)**. Study revealed that an administration of excess amount of Th1 type cytokines such as IL-2 or IFN-γ induced abortion in mice, and stimulation of toll-like receptor induced Th1-type cytokine production, resulted in abortion **(Lin *et al.,* 2009).**

TGF-β1 also is well known to be important for regulation of the cytokine network during pregnancy and to maintain a healthy fetus. TGF-β1 is present in trophoblasts and

is known to produce a number of cytokines, including IL-1β, TNF-α, IL-6, IL-8 as well as TGF-β1 **(Graham *et al.,* 1992).** In the endometrium TGF-β modulates epithelial proliferation, enhances gland formation, promote angiogenesis, activation and proliferation of fibroblasts, and deposition of extracellular matrices**.** TGF-β1 may be necessary to maintain pregnancy but also be a risk factor for recurrent miscarriages **(Ogasawara *et al.,* 2000).** IL-8, have been found in response to infection in pregnant women and have been correlated with adverse outcomes including chorioamnionitis and preterm labour. IL-8 may also play a fundamental role in cervical maturation in both normal and pathologic pregnancy. The increased cytokines production and further, COX-2 mRNA expression, and maximum prostaglandin synthesis prior to labour, shows that increased prostaglandin synthesis within fetal membranes precedes, and is therefore a cause, rather than a consequence, of labour.

Furthermore, endocrine factors like progesterone and estrogen have also been implicated in the etiology of spontaneous abortion, with poorly understood roles. In pregnancy, progesterone is in dynamic balance with estrogen in the control of uterine activity. Progesterone is critical for the survival of the pregnancy, until luteal-placental shift occurs at 7 to 9 weeks gestation **(Essah *et al.,* 2004).** It is important to promote endometrial decidualization by preparing the uterus for implantation of the blastocyst and in maintaining the pregnancy. There is increasing evidence that progesterone exhibits anti-inflammatory activities **(Piccinni *et al.,* 2001),** which might be beneficial in the prevention of pre-term birth **(Groom *et al.,* 2007)** and miscarriage. This endocrine cascade ultimately leads to both the activation and stimulation of the myometrium through the increased production of stimulatory PGs. Thus, low progesterone values are

associated with miscarriage and ectopic pregnancy, both considered as non-viable pregnancies.

Also, evidence suggests that the dysregulated extra-cellular matrix (ECM) proteolysis during the processes of tissue repair following infection and inflammation plays a key role in the development of fibrotic sequelae of chlamydial infection in humans. Matrix metalloproteinases (MMPs) are the family of proteolytic enzymes, play a central role in the breakdown of ECM. MMPs were found secreted by the pre-implantation human and canine embryo and maternal placenta **(Beceriklisoy *et al.,* 2007; Schafer-Somi *et al.,* 2008).** MMP-2 and -9 are found to be involved in rat embryo implantation and MMP-2 expression was also determined in gestational endometrium and placenta in goat **(Uekita *et al.,* 2004).** Improper turnover of endometrial ECM is associated with disturbances of implantation. Abnormalities in the remodelling of endometrial ECM have been found associated with bleeding disorders, abnormal histology and implantation defects **(Skinner *et al*., 1999)** and abnormality at the tissue or molecular level has been suggested in some cases of recurrent miscarriages and infertility **(Serle *et al*., 1994).** Biosynthesis of MMP-2 is significantly higher in the early stages of the pregnancy **(Kizaki *et al.,* 2008)**. MMP-2 has been suggested to be the key regulator of trophoblast invasion in early pregnancy **(Seval *et al.,* 2004)**. A recent study showed alterations in concentrations of proMMP-9 and TIMP-1 in serum and urine of pregnant women experiencing preterm or term uterine contractions **(Anumba *et al.,* 2010).** Expression level of MMP-9 and the MMP-9/TIMP-3 mRNA ratio was found to be associated with spontaneous abortion **(Jiang & Qi, 2015).**

Together with tissue inhibitors (TIMPs), MMPs form a balance to maintain normal early pregnancy and placental development. Tissue inhibitors for MMPs, such as TIMP-1 and TIMP-2, regulate protease activity. TIMP-3 is the major tissue inhibitor of MMP-9 among the four TIMPs and regulates the invasion process of cytotrophoblast cells **(Whiteside *et al.,* 2001)**, whereas TIMP-1 and -2 are expressed at relatively stable levels in proliferative and secretory endometrium **(Maatta *et al*., 2000)**. On the other hand, marked increase in the expression of TIMP-3 has been observed during the secretory phase, suggested its role in the preparation of endometrium for decidualization and implantation **(Maatta *et al*., 2000).**

In the light of above, extensive research that more research needs to be done on the contribution of the maternal, fetal and placental-decidual inflammatory responses to specific infections such as *C. trachomatis* during spontaneous abortion. It is thus hypothesised and envisaged that the study on the cyclooxygenases/ prostaglandin-signaling pathway along with pro-inflammatory cytokines, hormones (estrogen/ progesterone) and matrix metalloprotenases and their inhibitors will lead to an improved understanding of varied immunomolecular aspects during spontaneous abortion in women infected with *C. trachomatis* and will have implications in the prognosis and development of intervention strategies against subsequent miscarriage.

## 1.2. Review of literature

### 1.2.1. Overview

*Chlamydia trachomatis,* a gram negative, obligate intracellular bacterium is the most common sexually transmitted bacterial disease worldwide **(Howie *et al.,* 2011; Ziklo *et al.,* 2016).** Infection with *C. trachomatis* is a public health concern because of its chronic oligosymptomatic or asymptomatic course that poses a great clinical and epidemiological problem. These silent infections may lead to dangerous and frequently irreversible potentially devastating reproductive consequences in women including infertility and ectopic pregnancy **(Darville *et al.*, 2010),** pelvic inflammatory disease such as endometritis, cervicitis, and salpingitis **(Fatholahzadeh *et al.,* 2012).** *C. trachomatis* causes infection in the lower genital tract in women and men. Women chiefly carry the major burden of the disease and these women are also a potential source of infection to their partners, which can result in upper genital tract complications and transmission of infection during pregnancy and labour. Chlamydiae ascend into the upper reproductive tract where chronic infection can lead to silent or symptomatic inflammation resulting in mucopurulent cervicitis **(Brunham *et al.,* 1984),** acute urethral syndrome and tubal factor infertility **(Hafner 2015).** Mucopurulent cervicitis can lead to at least three type of complications **(Paavonen & Eggert-Kruse, 1999)** - ascending intraluminal spread of organism from cervix producing pelvic inflammatory disease **(Oakeshott *et al.*, 2010)**; ascending infection during pregnancy resulting in premature delivery, premature rupture of membrane, chorioamnionitis and neonatal infections (conjunctivitis and intestitial pneumonia); and also an increased risk of the development of cervical carcinoma

**(Paavonen & Eggert-Kruse, 1999)**. A 3- to 4-fold increased risk of transmission of HIV is an added cause of concern **(Weinstock *et al.,* 2004).** Other complications include ectopic pregnancy, endometritis and infertility etc. **(Brunham *et al.*, 2005).** *C. trachomatis* infection is a known risk factor for ectopic pregnancy and preterm birth (**Bakken *et al.,* 2007; Shaw *et al.,* 2011).** The colonization of *C. trachomatis* in the genital tract during early gestation has been associated with preterm birth, premature rupture of membranes, spontaneous abortion, perinatal morbidity and mortality in the pregnant women **(Rours *et al.,* 2011; Choi *et al.,* 2012).**

### 1.2.2. Classification & other types of *Chlamydia*

According to Bergey's manual's classification, *Chlamydia* belongs to the order Chlamydiales and family Chlamydiaceae and type *Chlamydia*. Type includes four species of *Chlamydia,* which are pathogenic to humans including *C. trachomatis*, *C. pneumoniae*, *C. psitaci* and *C. pecorum* **(Fig. 1.2).** They are characterised by a common antigen group and developmental cycle but they have different phenotypes and pathogenicity. Differences and common features are shown **(Table 1.1).**

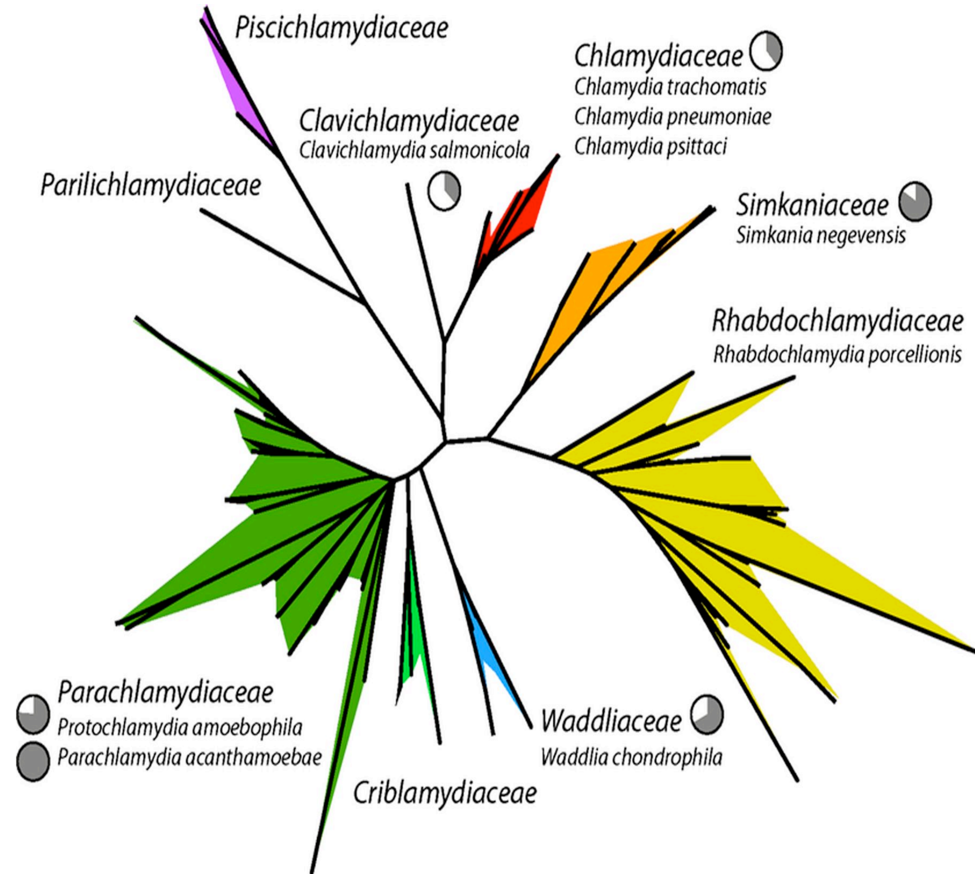



**Fig. 1.2:** The phylogenetic tree of Chlamydiae (*Omsland et al., 2014)*.

**Table 1.1:** Comparison of species of the family *Chlamydiaceae (Pawlikowska & Deptuła, 2012; Juszczyk & Samet, 2003).*

| Species (Gatunek) | *C. trachomatis* | | | *C. pneumoniae* | *C. psittaci* | *C. pecorum* |
|---|---|---|---|---|---|---|
| Biotype | Trachoma | LGV | mouse | TWAR | 1, 2, 3–9 | no data |
| Serotype | A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, K | L1, L2, L2a L3 | lack | TW-183, AR-37, AR-277, AR-388, AR-427, AR-231, LR-65 | probably 1 i 2 | no data |
| Natural host | human | | | human | animals | animals |
| Infection route | contact | | | inhalation | inhalation | inhalation |
| Place of infection | conjunctival and genitourinary epithelial cells | | | respiratory epi-thelium | respiratory epi-thelium | respiratory epithelium |
| Diseases | trachoma, venereal granuloma, infection of the urogenital system, inclusion conjunctivitis, reactive arthritis, conjunctivitis and pneu-monia in children | | | pneumonia | zoonoses | zoonoses |
| Number of serotypes | 18 | | | 1 | numerous | 3 |
| LPS antigen (Antigen LPS) | (+) | | | (+) | (+) | (+) |
| EB shape | round | | | round | pear-shaped | pear-shaped |
| Antibiotic sensitivity | (+) | | | (–) | (–) | (–) |

In this taxonomy, the two lines and a new species of the family Chlamydiaceae were identified. In the research of Everetti *et al.,* the new taxonomy of microorganisms of the family *Chlamydiaceae* is as follows; Order: Chlamydiales, Family: *Chlamydiaceae*, Type: *Chlamydia*, Species: *Chlamydia trachomatis*, *Chlamydia muridarum* sp nov., *Chlamydia suis* sp nov. Biotype: trachoma, LGV, type: Chlamydophila, Species: *Chlamydophila abortus* sp nov*., Chlamydophila psittaci comb nov*., *Chlamydophila felis* sp nov., sp nov *Chlamydophila caviae*, and *Chlamydophila pecorum* comb (**Pawlikowska & Deptuła 2012**). The term *Chlamydia trachomatis* derives from the Greek words chlamydos and trachoma. "*Chlamys*" – a coat, which are inclusions of intracellular bacteria flattening the host cell nucleus. "*Trachoma*"- an eye disease that leads to cicatricial changes, known as "rough eye" **(Markowska, 2002)**. Currently, *C. trachomatis* species consists of two biotypes: trachoma –C/PK-2 strain, and LGV – venereal granuloma with L2/434/BU strain. A mouse biotype belonging to this species has now been called *C. muridarum,* causes pneumonia in mice (**Pawlikowska & Deptuła 2012**)**.** Human pathogenic strains are divided into 18 serotypes **(Fig. 1.3).** The trachoma biotype includes A, B, Ba, and C serotypes. They are transmitted by indirect contact. D, Da, E, F, G, H, I, Ia, J and K serotypes, are responsible for oculogenital infections (pneumonia in children, conjunctivitis in children and adults, infections of urogenital system in adults). L1, L2, L2A and L3 – the LGV biotype causes inguinal lymphogranuloma venereum, inflammation of the rectum, genital ulcers, and infections of the lymphatic tissue **(Stefanow & Ostaszewska, 2000; Markowska, 2002; Pawlikowska & Deptuła, 2012**). Chlamydiae are microorganisms exhibiting common

features of bacteria and viruses. Nevertheless, since 1966 they have been classified as bacteria **(Markowska 2002; Heczko**, **2007**; **Pawlikowska & Deptuła, 2012)**.

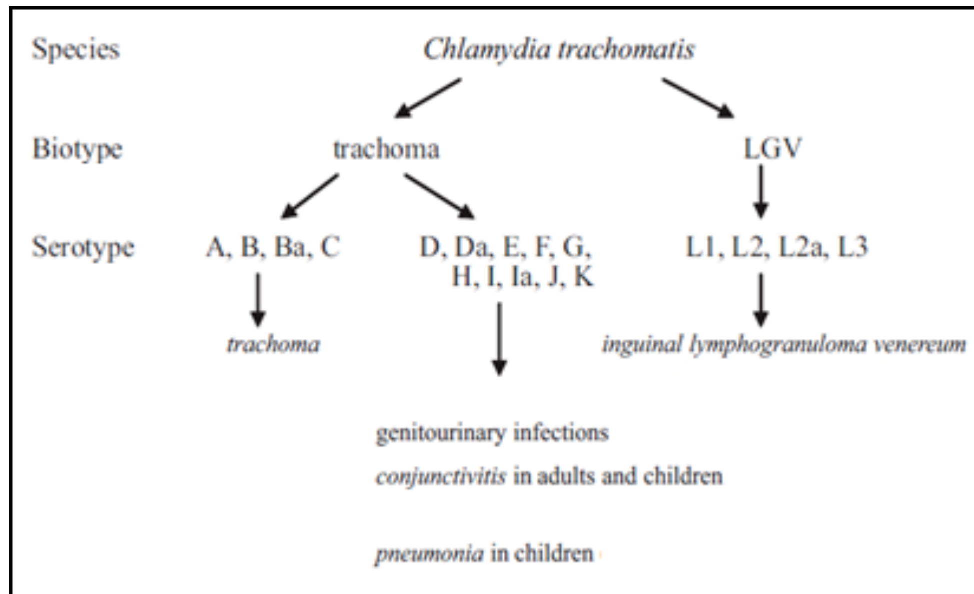



**Fig. 1.3:** Serological types of *Chlamydia trachomatis* and diseases caused by them *(Bielecki et al., 2004)*.

### 1.2.3. Developmental cycle of *Chlamydia trachomatis*

*Chlamydia*, an intracellular parasite of humans and animals is widespread in environment. What makes *C. trachomatis* different from other bacteria is its unique biphasic developmental life cycle, having the two coexisting morphological forms; extracellular infectious form, Elementary Bodies (EBs) and intracellular metabolic form, Reticulate Bodies (RBs) **(Table 1.2)**. EBs attach to epithelial cells, after which they are internalized into a membrane bound vesicle called an inclusion. It lasts from 24 to 48 hours. In its development cycle it uses the host cell metabolic pathways. EB can be

transferred from person to person. RB is non-infectious and has a reduced shell, formed after cell infection, and is capable of intracellular multiplication (inclusion bodies are created). The *Chlamydia* cell wall does not contain peptidoglycan, is thick, stiff, and similar to the wall of gram-negative bacteria. Disulfide bridges provide resistance of EB to mechanical and oxidative stress. The presence of hemagglutinin facilitates its adhesion and penetration into host cells, which probably also affects the infectivity against the host cell **(Markowska, 2002; Pawlikowska & Deptuła 2006, 2012).**

**Table 1.2:** The main differences between elementary and reticulate body *(Bielecki et al., 2004)*

| Property | EB | RB |
|---|---|---|
| Size | 0.2–0.4 μm | 0.8–1.3 μm |
| Morphology | – lancet-shaped,<br>– high electron density,<br>– the presence of a thick, rigid cell wall with large amounts of cysteine protein,<br>– the presence of cytoplasmic membrane | – oval-shaped,<br>– low electron density,<br>– the presence of a thin membrane with high permeability |
| Haemaglutinin in the cell wall | present | lack |
| Infectivity | high | lack |
| Place of development | extracellular (mainly) and intracellular | intracellular |
| Sensitivity to:<br>– digestion by trypsin,<br>– mechanical stress,<br>– oxidative stress | resistant | sensitive |
| Metabolic activity | lack | active |
| The ability to reproduce | lack | able |

The developmental cycle can be divided into several stages **(Fig. 1.4).** An infection occurs when EB bind to sensitive cells (columnar epithelial cells of conjunctiva and genital organs) and a reversible followed by irreversible connections are produced. The next stage is the transition of EB into the cell through endocytosis and producing a common phagosome together with other EBs, which are called cytoplasmic inclusions (after 4–8 hour) **(Workowski & Berman, 2008)**. EB differentiates into RB (after 8–12 hour), while a reduction of the disulfide bridges occurs, leading to modification of structure of outer membrane proteins **(Zdrodowska-Stefanow & Ostaszewska, 2000)**. Within the phagosome, RB replication occurs (several divisions), which is associated with a significant enlargement of the cytoplasmic inclusion. It fills the cytoplasm therefore the cell nucleus is pushed to the cell perimeter. After about 36 hours following infection, RBs are surrounded by a plasma membrane and undergo transformation to EB. After 48 hours following cell infection, EB is released through lysis or cytoplasmic inclusion rupture without damaging the host cell. The 50–1000 mature bodies that exist outside the cell are the source of infection for other host cells **(Oliveira *et al.,* 2008).**

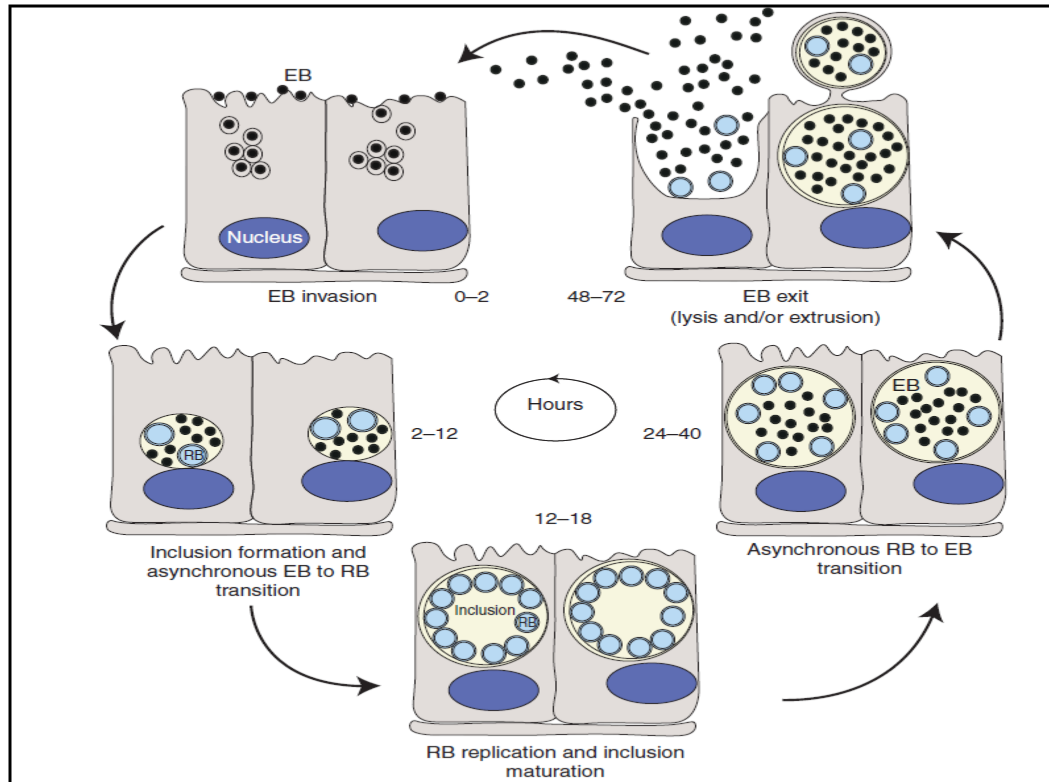



**Fig. 1.4:** Life cycle of *Chlamydia (Hatch, 1999).*

### 1.2.4. Characteristics of *Chlamydia trachomatis* antigens

*C. trachomatis* is a strong immunogen that stimulates immunological processes of the host. At present, 4 antigen groups are distinguished within the *Chlamydia* genus: group-specific, species-specific, type-specific and subspecies-specific **(Fig. 1.5)**. **(i).** Group-specific antigen, shared by all *Chlamydia* species, the best is known. Its main component, a thermostable polysaccharide complex, contains two constituents: lipopolysaccharide (LPS) and glycolipid (GLXA). LPS is present on the surface of EB and RB during the developmental cycle of *Chlamydia.* GLXA is located on the surface of EB and RB within the cell membrane of cytoplasmic inclusions. **(ii).** Species-specific antigens, varying according to the *Chlamydia* species, have a protein

structure and are thermolabile. The most important are the major outer membrane protein (MOMP) of 38–42 kDa, constituting 60% of all outer membrane proteins; and the so-called chlamydial heat shock proteins (c-HSP) of 10 and 60 kDa (Ward, 1983). MOMP acts as a structural protein and is involved in the differentiation of the chlamydial development cycle. It is a protein with highly immunogenic properties, containing epitopes specific to the genus, species, subspecies and serotype. The c-HSP60 antigen is moderately immunogenic in comparison to the poorly immunogenic smaller protein of 12 kDa molecular weight. They are invisibly present on the surface of the membrane, and antibodies specific to these proteins do not bind to live EB. **(iii).** The third antigen group is of type-specific antigens, characteristic of the respective serotypes within the *C. trachomatis* species, most likely polypeptides of 30 kDa and thermolabile by nature, placed on the surface of EB and RB**. (iv).** The last type of antigen, also of polypeptide structure, refers to subspecies-specific antigens **(Wang & Grayston, 1991).**

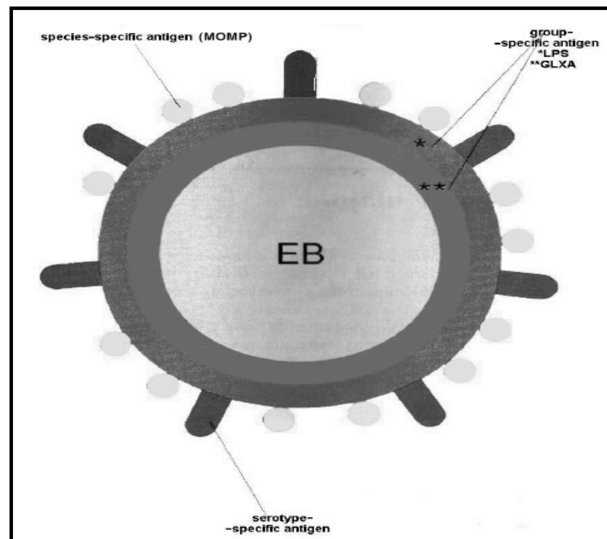



**Fig. 1.5:** Antigenic structure of *Chlamydia trachomatis (Zdrodowska-Stefanow et al., 2003).*

### 1.2.5. Epidemiology of *Chlamydia trachomatis* infection

- **International status**

Sexually transmitted infections are a major global health problem with an estimated 340 million new cases of “curable” infections occurring each year worldwide **(WHO, 2007)** and the prevalence of the disease was so high that it estimated as 101 million new cases in 2005 worldwide **(WHO, 2015).** The bacterium *C. trachomatis* is the etiological agent of the most common sexually transmitted infection in North America and Europe **(Finethy & Coers, 2016).** *C. trachomatis* genital infection is often asymptomatic in over 70% of cases as a result few population-based prevalence or incidence estimates is available. The most prevalent bacterial STIs are those caused by *C. trachomatis*. The incidence of *C. trachomatis* infection has increased during the past 10 years **(Bebear & Beyrberac, 2009).** *C. trachomatis* is estimated to infect ~ 2.8 million people in the United States and over 90 million people worldwide each year **(Weinstock *et al.,* 2004; MMWR, 2014).** The highest incidence is among young women between the ages of 15 and 24 **(Lee *et al.,* 2001).** The incidence and prevalence of *C. trachomatis* in south-east Asia region was reported as 7.2 million and 8 million, respectively by World Health Organization **(WHO, 2015, Fig., 1.6, 1.7).**

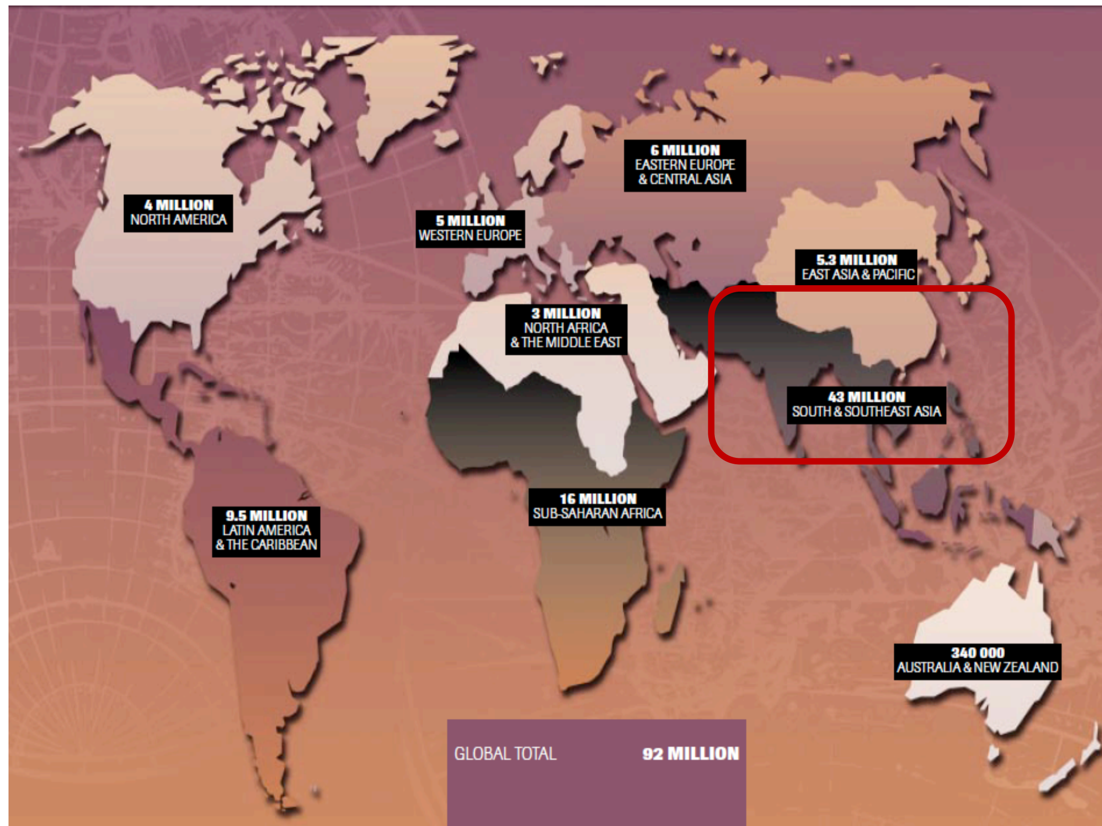



**Fig 1.6:** WHO report on global prevalence & incidence of curable sexually transmitted diseases (2002).

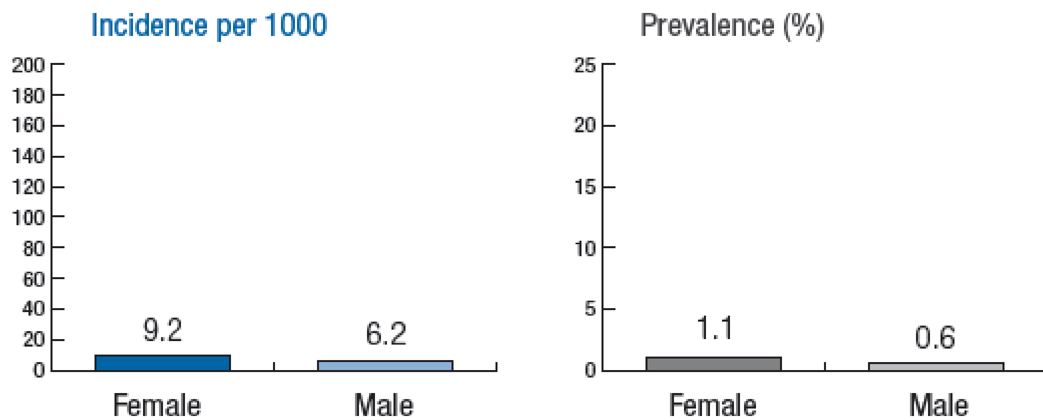



**Fig 1.7:** WHO South-East Asia Region (Highlighted) comprises 11 countries with an estimated population in 2008 of 945.2 million adults between the ages of 15 and 49. Incidence is 7.2 million cases of *C. trachomatis and* 8.0 million adults were infected with *Chlamydia trachomatis.* Graphs showing; the incidence and prevalence of *Chlamydia trachomatis* in female & male, WHO 2012)

*C. trachomatis* has been associated with spontaneous abortions though not consistently. Several studies have reported a higher prevalence of *C. trachomatis* antibodies in spontaneous **(Baud *et al.,* 2007)** or recurrent **(Baud *et al.,* 2008)** abortions. Miscarriage could be induced by persistent *C. trachomatis* infection spreading to the foetal tissues or endometrium. Many patients exhibited *C. trachomatis*–positive serologic results without *C. trachomatis* DNA suggesting that miscarriage occasionally be induced by the past chlamydial infection or persistent *C. trachomatis* antibodies that interfere with embryonic antigens **(McDuffie *et al.,* 1992).** In one study, 21% women from couples with spontaneous abortions consulting a reproductive medicine centre tested positive for *C. trachomatis* by direct immunofluorescence compared to 9% women without spontaneous abortions and term pregnancies ('$p$' < 0.05). The infection rate increased to 69% ('$p$' < 0.001) when both partners of the couples were considered **(Vigil *et al.,* 2002)**. Various models for studying the pathogenesis of *Chlamydia*-related spontaneous abortions have been proposed, being either direct zygote infection or an immune response to heat shock proteins expressed by the zygote that is triggered by previous *C. trachomatis* infection, and reactivation of latent chlamydial infection or endometrial damage from past chlamydial infection **(Vigil *et al.,* 2002).** 31% prevalence of *C. trachomatis* was found in the serum of spontaneous aborters by ELISA **(Witkin & Ledger, 1992)**. Chlamydial antibodies were also detected in women who suffered miscarriage **(Osser *et al.,* 1996).** In a study from Iran, 21% *C. trachomatis* infection was found by Polymerase chain reaction (PCR) in endocervical smears of women with abortion **(Zeighami *et al.,* 2008).** Other studies have found varied *C. trachomatis* prevalence in different clinical samples and different diagnostic methods such as; 12% by

PCR in Cervical swabs, 15% in products of conception/ cervicovaginal swab/ serum by using Real time PCR/ ELISA/ IHC and 32% in Endometrial curettage by PCR respectively **(Magon *et al.,* 2005; Trojniel *et al.,* 2009; Baud *et al.,* 2011).** Furthermore, women undergoing surgical or medical termination of pregnancy have been reported to have *C. trachomatis* infection with prevalences as high as 17% **(Renton *et al.,* 2006; Baczynska *et al.,* 2008).**

- **Indian status**

*C. trachomatis* has been termed a 'silent epidemic' that infects the columnar epithelial cell lining the endocervix. The prevalence upto 81% has been reported from India for asymptomatic genital chlamydial infection **(Bhujwala *et al.,* 1982; Mittal *et al.,* 1995).** The available Indian data show a wide variation in *C. trachomatis* prevalence and methods of laboratory confirmation. Some studies have found infection rates of Indian women ranging from 3.3% to 33% depending on the population sampled **(Alexander *et al.,* 1993; Mania-Pramanik *et al.,* 2001; Rastogi *et al.,* 2003; Singh *et al.,* 2003; Joyee *et al.,* 2004, 2005; Malik *et al.,* 2009; Becker *et al.,* 2010).** Most of these studies focus on high risk groups like STD/ infertility patients, female sex workers, and HIV positive women) and were limited by small sample sizes **(Singh *et al.,* 2003; Becker *et al.,* 2010)**. In Tamil Nadu, it was found that the prevalence of active genital *C. trachomatis* infection in urine of a healthy adult female population by nucleic acid amplification tests (NAAT) was 1.1% (95% CI: 0.4%–1.8%) **(Joyee *et al.,* 2004)** however, among symptomatic men and women attending a STI clinic, the prevalence of confirmed *C. trachomatis* infection by culture and/or nested- PCR detecting major outer membrane protein (MOMP) was

30.8% **(Joyee *et al.,* 2005).** The prevalence of *C. trachomatis* in pregnant women in India has been shown to vary by geographic region. In a study conducted in Vellore, India in 1993, prevalence of *C. trachomatis* in pregnant women was found to be 3.3% (95% CI: 1.2%–5.4%) using an enzyme immunoassay (EIA), which detects the presence of chlamydia antigen. A higher prevalence of *C. trachomatis* was also reported in rural women (5.9%) compared to urban women (1.8%) **(Alexander *et al.,* 1993).** Study from New Delhi found the prevalence of *C. trachomatis* infection in mid-pregnancy and at labor using the Chlamydiazyme H test to be 17% and 18.6% respectively **(Paul *et al.,* 1999).** In addition, study also found infants born to infected mothers experiencing conjunctivitis than those born to non-infected mothers, 12.5% versus 2.8% ($p = 0.04$) respectively **(Paul *et al.,* 1999).** Rastogi *et al.* also found *C. trachomatis* prevalence of 18.8% (95% CI: 14.76 –22.96%) among 350 pregnant women in New Delhi diagnosed using PCR and DFA **(Rastogi *et al.,* 2003).** In the study from our group, 15.6% *C. trachomatis* positivity was found by ELISA **(Rastogi *et al.,* 2000)** in women undergoing spontaneous abortion. Another study on prevalence of *C. trachomatis* infection in women with first trimester pregnancy losses showed 26% positivity **(Avasthi *et al.,* 2003).**

### 1.2.6. Immunopathology and infection caused by *Chlamydia trachomatis*

*C. trachomatis* is a strong immunogen which stimulates the immune system. Specific, nonspecific, humoral and cellular response mechanisms are involved in the course of *C. trachomatis* infection. In the genital tract of women, primary site of infections is columnar epithelial cells of endocervix and the urogenital epithelia of men **(Brunham & Rey-Ladino, 2005).** In men, ascending infection can cause prostatitis and epididymitis **(Cunningham & Beagley, 2008).** In women, the development of

*Chlamydia* disease sequelae is associated with the movement of *Chlamydia* from lower reproductive tract to the upper reproductive tract (URT). Mechanisms that lead to this ascension are not fully understood. It is thought that *Chlamydia* can gain access to the URT of women by attachment to sperm **(Vigil *et al.,* 2002).** It is also possible that movement along the reproductive tract is from general flow of fluids.

- **Primary infection**

In primary infection, non-specific immunity plays a special role, which includes local inflammation and the mechanisms associated with the anatomic barrier. It usually manifests itself as urethritis in men and/or cervicitis in women **(Zdrodowska-Stefanow & Ostaszewska, 2000).** Its course is most frequently oligosymptomatic or asymptomatic. In 24–48 hours after the infection, phagocyte infiltration of infection site occurs, mainly by polymorphonuclear leukocytes and monocytes. The presence of exudative fluid containing anti-microbial substances facilitates the destruction of microorganisms. During primary infection, a serial infection of mucosal cells may occur. The infected epithelial cells secrete various proinflammatory cytokines and chemokines including TNF-α, IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and growth regulated oncogene **(Rasmussen *et al.,* 1997)** Contrary to the rapid secretion of cytokines in infections induced by *Chlamydia,* the secretion is delayed up to 20–24 hour after infection onset. The released cytokines cause vasodilation, increased endothelial permeability, activation and migration of neutrophils, monocytes, macrophages and T lymphocytes, elevated expression of adhesion molecules and stimulation of cells for secreting cytokines. The local inflammatory reaction is most intense on 2–4 days after infection onset. A maximum concentration of TNF-α release a

strong initiator of inflammatory process (**Rasmussen *et al.,* 1997)** is observed 48 hour after infection onset and persists for 4 days after which it is reduced. At the same time, *Chlamydia* pass via lymphatic vessels to local lymph nodes, which in many cases become enlarged.

Some EBs released by decaying epithelial cells, are phagocytized by neutrophils, probably through the fusion of phagosomes and granulocyte lysosomes and the formation of the so-called phagolysosomes. T lymphocytes, mainly Th1 cells play an important role during the early phase of infection, which, due to *Chlamydia* antigen-induced activation, secrete, IFN-γ, necessary for infection regression **(Lampe *et al.,* 1998)**. IFN-γ increases the potential of various phagocytes, especially monocytes and macrophages, to destroy *Chlamydia* and stimulates the secretion of other cytokines, including IL-1. IL-1, in turn, by stimulating the secretion of IL-2 by Th1 lymphocytes, causes increased replication of cytotoxic lymphocytes and natural killer cells. The production of secretive IgA antibodies also plays a role in the neutralization of primary infection. Locally produced antibodies limit the spread of chlamydial infection, but do not eliminate the bacteria completely. The immune response of the host to the primary infection is transitory in most of the cases and is not associated with tissue damage.

- **Chronic Infection, recurrent infection, reinfection**

Recurrent infection and chronic infection associated with the persistence of bacteria into the host cells is more vicious. The inflammatory state develops in a considerably shorter time and is more intense, which is, associated with the presence of specifically sensitized Th1 lymphocytes. A recurrent stimulatory action of *Chlamydia* antigens results in a delayed type hypersensitivity. The delayed type reaction develops

within 24–48 hour due to antigen interaction with sensitized Th1 lymphocytes. These immune reaction processes lead to fibrosis, tissue damage and cicatrization within the affected organs. The outcome can be so severe, e.g. trachoma (conjunctiva, loss of sight), pelvic inflammatory disease (PID) leading to infertility, chronic pelvic pains, ectopic pregnancy and chronic urethritis **(Zdrodowska-Stefanow & Ostaszewska, 2000).** Since chlamydial infection is frequently asymptomatic, the sequelae are common and pose serious health and economic problems. Chronic infection may result from lack of treatment or improper therapeutic management. Certain cytokines, e.g. IFN-γ, TNF-α and TGF-β, play a significant role **(Ault *et al.,* 1996)**. They lead to disorders in the developmental cycle of the bacteria, including RB maturation delay or inhibition of their differentiation into infectious EB and, thus, to the growth of atypical forms of *Chlamydia.* The atypical forms are larger, non-infectious, have reduced metabolic activity, and do not replicate, yet remain alive. They display a different antigenic structure, characterized by a reduced number of MOMP and LPS and invariable amounts of the pathogenic cHSP-60, a strong stimulator of immune reactions **(Laverda *et al.,* 1999; Fig., 1.8).**

Irreversible sequelae occur mainly due to the delayed hypersensitivity reactions induced by this strong antigen or as a result of an autoimmune reaction associated with a 50% homology between the chlamydial and human HSP **(Witkin *et al.,* 1994).** In patients with complications, a decrease in the production of anti-MOMP antibodies with protective properties and an increased production of pathogenic anti-C-HSP antibodies are frequently observed. Reinfection is due to repeated infection, while recurrence is caused by the presence of a *Chlamydia* reservoir in the lymph nodes and spleen. Determination of the serotypes of patients infected with *C. trachomatis* and their partners

can help differentiate between these two cases. Reports on a large number of recurrences (20–39%) observed both in men and in women with chlamydial infection a few months after therapy may sound alarming **(Jones, 1990).** A special role in disease recurrence has been ascribed to macrophages, in which *Chlamydia* can persist even for 10 days. They circulate with macrophages round the body, finding a temporary shelter in the lymph nodes, spleen and serous cavities. *C. trachomatis* infections of the urogenital tract affect mainly young people. The less common spread of the infection in older people may be caused by low exposure to *Chlamydia* and by physiological changes, which reduce sensitivity but, on the other hand, it can be due to resistance acquired during natural exposure.

### 1.2.7. Laboratory diagnosis of *Chlamydia trachomatis*

The clinical spectrum of human chlamydial infections includes clinically unapparent infection, acute symptomatic infections as well as persistent infections (defined as the presence of viable but non-cultivable chlamydiae). Asymptomatic nature of the disease and the increasing spectrum of infections caused by *C. trachomatis* emphasize the need for the sensitive and reliable laboratory methods. NAATs are the latest diagnostic methods and have replaced culture as the method of choice to diagnose chlamydial infection. However, various laboratory tests, culture and non-culture methods, are available and may still be used to diagnose *C. trachomatis* infection **(Black, 1997; Van de Laar, 2009) (Table 1.3).** The sensitivity of these tests depends on several factors: the nature of the disease and subsequent site of specimen collection, the quality of the specimen and transport medium, the intrinsic quality of the test and the precision with which the test is carried out.

- **Collection of clinical specimens**

Proficiency in specimen collection and transport is paramount to accuracy in diagnostic testing. Sensitivity and specificity of diagnostic tests for *C. trachomatis* have shown to be related with adequacy of the specimen. As the chlamydiae are intracellular, the host cells that harbour the organism should be included in the specimen collection, especially in methods with involves the direct study of organism. The choice of sampling sites can influence the likelihood of recovering the pathogen. Endocervical swab, vaginal/introital swab, vulval swab as well as urethral and rectal swab and first void urine are the common samples taken from the female patients. Placental tissues, chorionic villus and endometrium were also used to detect *C. trachomatis* infection. The likelihood of isolation is optimized if the specimen is refrigerated immediately after collection at 2-8˚C. The time between sample collection and processing should be less than 48 hour, or the samples may be frozen at -70˚C until processed. To preserve the viability of *Chlamydia* in specimen, use of foetal bovine serum is helpful. Two-molar sucrose phosphate or sucrose glutamate phosphate are the most commonly used transport medium.

- **Non-molecular diagnosis of *Chlamydia trachomatis* infection:**

**a. Cytology**

Cell scrapings can be stained with iodine and examined for the presence of typical intracytoplasmic inclusion bodies. Since none of the other *Chlamydia* species contains glycogen, which stains with iodine, the finding of iodine-stained inclusion bodies is specific for *C. trachomatis* **(Chiappino *el al.,* 1995)**. This method is not as sensitive as

the other methods to diagnose *C. trachomatis* infection, but still exceeds 60% when diagnosing neonatal conjunctivitis.

**b. Culture**

Culture has been considered the gold standard for many years, and still is the method of choice for medico-legal issues and antibiotic susceptibility testing. Cell culture is the only method to detect viable organisms and therefore, highly specific to diagnose *C. trachomatis* infection **(Black, 1997).** *C. trachomatis* is able to grow when specimens are brought to 37°C, inoculated onto the surface of confluent monolayers of susceptible cells (McCoy, Hela or BHK lines). After 48-72 hours incubation of the infected cell monolayers, infected cells can be examined for growth of inclusion bodies, which may be microscopically visualised. Culture has several disadvantages.

**c. Antigen detection by enzyme immunoassay (EIA) technique**

EIA techniques can be used to detect *C. trachomatis* **(Bakir *et al.,* 1989).** The specimen is incubated with an antibody to detect chlamydial LPS on EBs membrane, an enzyme substrate is also added which gives colour and can be visualized microscopically or measured by spectrophotometry. EIA has several disadvantages and is less sensitive than cultures and NAATs (40% to 75%).

**d. Direct fluorescent antibody (DFA) detection**

For DFA, the specimen is smeared on a slide, air dried, fixed and stained with monoclonal antibodies labeled with fluorescein that further binds to MOMP of EBs present in the specimens, which produces brightly fluorescing and morphologically distinctive particles for direct visualization by fluorescence microscopy. DFA is highly

specific (99%) compared to culture with a sensitivity of 75% to 85% **(Svensson *et al.,* 1991).**

**e. Serology diagnosis**

Serology has been an important technique in epidemiological studies, describing clinical spectrum of chlamydial persistent infections. Serological tests have limited value in diagnosing *C. trachomatis* infection because tests do not distinguish current from past infection but it may be helpful in diagnosing anti-chlamydial antibody in pregnant women and in spontaneous aborters. Detection of *C. trachomatis* IgG and IgA antibodies are studied in several studies **(Rastogi *et al.,* 2000; Trojniel *et al.,* 2009).**

- **Molecular diagnosis of *Chlamydia trachomatis* infection**

NAATs have really improved the detection of *C. trachomatis* infections and offers high sensitivity and specificity. NAATs are based on the amplification and detection of specific DNA or RNA nucleic acid sequences unique to *C. trachomatis* in specimens. NAATs use different target, probe or signal amplification technologies such as PCR, strand displacement amplification, transcription-mediated amplification, nucleic acid sequence based amplification, ligase chain reaction, or hybrid capture. NAATs are highly specific (99% to 100%) and have a high sensitivity of 90-95%, which is higher than any methods **(Van Dyck *et al.,* 2001).** The most commonly used NAATs at the moment are the BD Probe Tec, Cobas Amplicor, Cobas TaqMan, and Aptima.

PCR is also appropriate for diagnosis of *C. trachomatis* infection in persistent phase. PCR has been used in several studies to detect *C. trachomatis* infection in various disease groups like infertility, spontaneous abortion and arthritis etc. **(Rastogi *et al.,***

**2000, Kumar *et al.,* 2014).** Furthermore, PCR can be used with non-invasive specimens such as urine or vulvovaginal swabs **(Watson *et al.,* 2002)**. The main disadvantages of NAATs are the costs and the reduced performance if inhibitors such as blood, mucus etc. are present in specimens **(Mahony *et al.,* 1998).** The burden of *C. trachomatis* organisms in the genital tract can be detected by quantitative real time PCR and can vary from 10 to more than a million organism/ ml of sample **(Michel *et al.,* 2007).**

**1.2.8. Spontaneous abortion**

Spontaneous abortion is pregnancy that ends spontaneously before the fetus has reached a viable gestational age (24 weeks) **(Rai & Regan, 2006)**. It is one of the most common yet under-studied adverse pregnancy outcomes. In the majority of cases the effects of spontaneous abortion on women's health are not serious and may be unreported. However in most serious cases symptoms can include pain, bleeding and a risk of haemorrhage. A small number (0.5–1%) of women wishing to have children may experience three or more successive abortions, a condition known as 'recurrent abortions' **(Bulletti *et al.,* 1996).** Although most abortions are sporadic (single), recurrent abortions (three or more consecutive losses) are observed in 1% of couples **(Rai & Regan, 2006)**. Spontaneous abortions can further classified into two more types; complete abortion is when all of the products (tissue) of conception leave the body however in incomplete abortion, some of the products of conception leave the body and some retains inside the uterus. Spontaneous abortions are common during the first trimester (up to 40% of pregnancies) and can be occurred so early that mother remains unaware of her pregnancy. Second trimester loss is less common, occurring in only 1–2% of pregnancies.

**1.2.9. Etiology of spontaneous abortion**

The reasons of abortions are often unknown. In ~ 50% of early miscarriages the fetus exhibits chromosomal abnormalities in chromosomal numbers and in structures **(Suzumori & Sugiura-Ogasawara, 2010).** Several other factors have been associated with increased risk of miscarriage like genetic, endocrine, infectious, autoimmune etc. **(Fig. 1.8).** Parent's age also has a significant role in adverse pregnancy outcome and the risk is increases if the parents are 35 years or more old and if the mother age is 42 years, the risk is 50% higher **(Maconochie *et al.,* 2007).** In addition factors such as psychological state of women, ethnic origin, very low/ high pre-pregnancy BMI, stress, use of anti-inflammatory drugs, smoking and alcohol consumption have also been found associated with higher rates of miscarriage **(Maconochie *et al.,* 2007).** It has been reported that women with prior history of miscarriage are at a higher risk of second pregnancy loss **(Kashanian *et al.,* 2006).** Furthermore, a number of infections have also been found associated with miscarriage **(Benedetto *et al.,* 2004)**, stillbirth **(Goldenberg & Thompson, 2003)** and preterm delivery **(Garland *et al.,* 2002).** 15% of early and 66% of late miscarriages has been attributed to infections **(Baud *et al.,* 2008)**. In a recent study, 78% of 101 tissue samples from miscarriage were infected with bacteria (chorioamnionitis) **(Allanson *et al.,* 2010).**

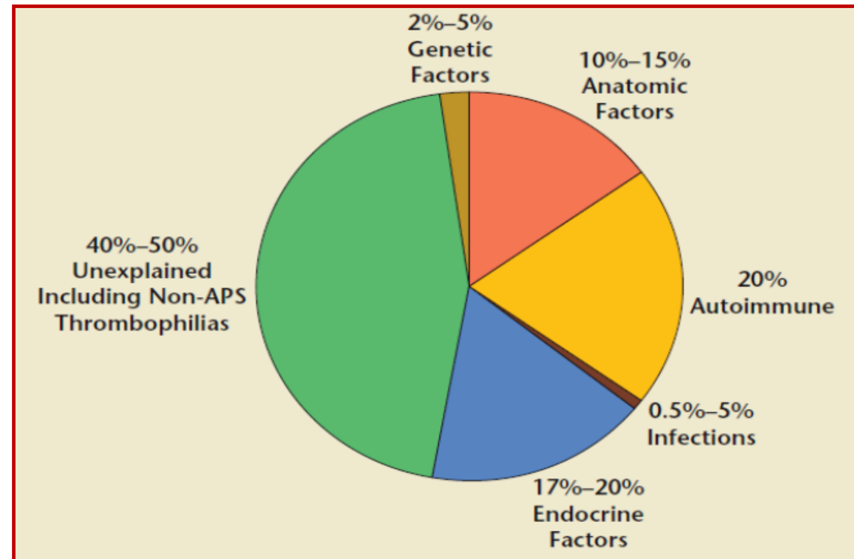



**Fig. 1.8:** Etiology of abortions. APS; antiphospholipid antibody syndrome *(Ford & Schust, 2009)*

**1.2.10. Association of other pathogens with spontaneous abortion**

Genital tract infections may play an etiologic role in the occurrence of preterm labor and spontaneous abortion. The most common pathway of intra-amniotic infections is the ascending route, and both lower and upper genital tract infections may be involved **(Soper *et al.,* 1989)**. Bacterial vaginosis has been predominantly associated with late miscarriages **(Hay *et al.,* 1994)**. A significantly higher frequency of Bacterial vaginosis among women with a history of at least one late miscarriage (21%) than among those with recurrent early pregnancy loss only (8%) was also reported **(Llahf-Camp *et al.,* 1996).** Spontaneous abortion and the presence of *Gardenerella vaginalis, Mycoplasma hominis, and Ureaplasma urealyticum* were also associated with subsequent miscarriage **(Donders *et al.,* 2000).** A retrospective review suggested an association between Group

B *Streptococcus* and miscarriage: Group B *Streptococcus* was the most significant pathogen in spontaneous abortions, being often the sole pathogen equally recovered in women both with intact and ruptured membranes. The genital mycoplasmas, *U. urealyticum* and *M. hominis* may be transmitted by direct contact between hosts vertically from mother to offspring. These microorganisms are identified frequently in the amniotic fluid of women in preterm labor and have been associated with increased risk of recurrent pregnancy losses **(McDonald *et al.,* 2000).** *U. Urealyticum* was found significantly high from products of conception of early and mid-trimester abortions in patients with spontaneous and recurrent abortions **(Sompolinsky *et al.,* 1975).** *Treponema pallidum* was also reported to be associated with spontaneous abortion and were at risk of repeated abortions **(Wendel *et al.,* 1988).** TORCH associated microorganisms were also found to be associated with spontaneous abortion. *Toxoplasma gondii* was also found in women with a history of miscarriage **(Alvarado-Esquivel *et al.,* 2014).** Cytomegalovirus, which can produce chronic or recurrent maternal infection, is the most implicated virus as a potential cause of RSA. In both primary and recurrent maternal infection, Cytomegalovirus can reach placenta with subsequent tissue damage, and transmission to fetus during pregnancy **(Nigro *et al.,* 2011).** Herpes simplex virus was also found positively correlated with spontaneous abortions **(Kapranos *et al.,* 2009).**

### 1.2.11. Pathogenic mechanisms for induction of abortion by infections

An etiologic role in the occurrence of abortion, preterm labor and premature rupture of membranes is played by genital tract infections. All microorganisms can produce acute infection and further abortion, but only a few can cause chronic disease capable of causing recurrent abortion. The pregnant woman and her fetus are susceptible

to many infections **(Goldenberg & Thompson, 2003).** Some of these may be life threatening for the mother, whereas others may have a profound impact on the fetus. Genitourinary tract or systemic infection with gram-negative bacteria in pregnant women can cause abortion and several other perinatal complications **(Penta *et al*., 2003).** The main mechanisms by which infections induce abortion include:

(a) Production of toxins or cytokines (i.e. TNF-α), which induce uterine contractions or damage the feto-placental unit

(b) Fetal infection, resulting in fetal death or life-threatening malformations

(c) Placental infection, with subsequent placental insufficiency and fetal death

(d) Endometrial chronic infection, interfering with embryo implantation

(e) Amnionitis, which causes abortion in the first trimester as well as preterm labor in the third trimester. **(Fig. 1.9)**

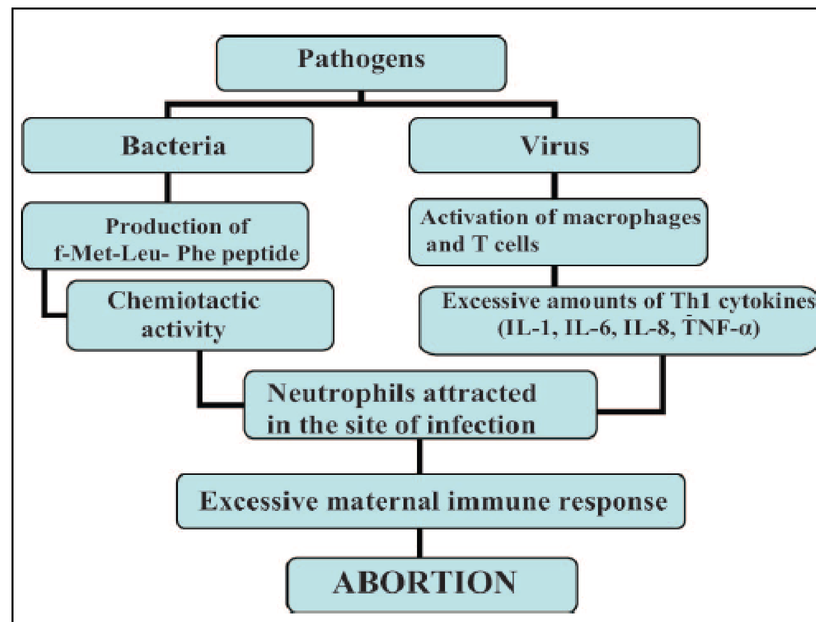



**Fig. 1.9:** Pathogenic mechanisms for induction of abortion by infections *(Nigro et al., 2011).*

### 1.2.12. *Chlamydia trachomatis*-induced spontaneous abortion

*C. trachomatis* infection is a major risk for the reproductive health in women and has been implicated in a substantial proportion of cases of subfertility and adverse pregnancy outcomes. Many studies are based on *C. trachomatis* recovery from urine, cervical smear and products of conception **(Rastogi *et al.,* 2000; Oakeshott *et al.,* 2002).** An increased prevalence of *C. trachomatis* antibodies in sporadic **(Vigil *et al.,* 2002)** or recurrent **(Quinn *et al.,* 1987; Witkin *et al.,* 1992)** miscarriages has been reported. An association between *C. trachomatis* infection and spontaneous abortion was found by other groups **(Wilkowska-Trojniel *et al*., 2009; Jahromi *et al*., 2010)**. In one study, positive IgG serology for *C. trachomatis* was associated with recurrent early miscarriages (<12 weeks of pregnancy) **(Baud *et al.,* 2007).** Thus, past or chronic *Chlamydia* infections might increase the risk for miscarriage, even in the absence of a detectable current infection, potentially by triggering chronic inflammation.

### 1.2.13. Immunopathogenicity of *Chlamydia trachomatis* induced spontaneous abortions

*C. trachomatis* is known to uniquely take a chronic course, an event which is conceivably required for the protracted host-pathogen interaction and thus establishment of pathology. This initiation of pathology has been associated with inflammatory damages related with seropositivity of chlamydial HSP-60 (cHSP-60) **(Hillier *et al.,* 1988).** Current or past infection with *Chlamydia* species may cause adverse pregnancy outcomes via two potential pathogenic mechanisms that affect the integrity of the placenta. Inflammatory activation of endometrial immunocytes by infections can cause

maternal immune response towards trophoblastic invasion and induce pregnancy failure in women. The immunological hypothesis postulates that the anti-*Chlamydia* immune response results in endometrial, placental and fetal damage. The cellular hypothesis suggests that cytokine release by persistently infected cells may directly cause pregnancy termination **(Fig. 1.10)**.

Cell-mediated immunity and associated IFN-γ production restrict the growth of *Chlamydia* **(Fig. 1.10).** IFN-γ induces indoleamine-2,3-dioxygenase (IDO), resulting in tryptophan depletion **(Brunham & Rey-Ladino, 2005).** Decreased tryptophan availability leads to differentiation of *Chlamydia* into noninfectious, nonreplicating aberrant bodies **(Brunham & Rey-Ladino, 2005)**, explaining the observed persistence of *Chlamydia* in the genital tract for prolonged periods after initial infection, even after effective antibiotic treatment. One of the characteristics of fetal–maternal immunity is its relative deficit in cell-mediated immunity, with reduced IFN-γ production, its production by T cells at the site of infection is likely to diminish as the *Chlamydia* antigen level decreases. In such conditions, the aberrant forms may be reactivated **(Fig. 1.10),** resulting in the release of infectious elementary bodies, which may lead to endometrial, placental, or fetal tissue damage. Further factors render the relationship between T cells, IFN- γ, IDO, tryptophan and *Chlamydia* even more complex. For instance, *Chlamydia* can synthesize tryptophan, with indole provided by the local microbial flora of the female genital tract. Also, human trophoblast cells constitutively express IDO, which leads to tryptophan depletion, peripheral T-cell tolerance and maternal acceptance of fetal allograft **(Brunham & Rey-Ladino, 2005).**

Another attractive mechanism involves the induction of an autoimmune response to an epitope shared by a chlamydial and a fetal antigen **(Fig. 1.10) (Witkin *et al.,* 1992; Karinen *et al.,* 2005).** Indeed, persistent infection with *C. trachomatis* produces high levels of a chlamydial 60 kDa heat shock protein (c-HSP60), against which the infected women may be sensitized. Expression during pregnancy of a human 60 kDa heat shock protein (h-HSP60), which shares a 50% amino acid sequence homology with the c-HSP60, can reactivate the anti-c-HSP60 immune response and may lead to rejection of the embryo.

*Chlamydia* infection elicits inflammatory responses that may cause pregnancy loss by giving rise to a detrimental combination of cytokines. Indeed, inflammatory cytokines such as IFN- γ, TNF-α and IL-2 have an abortigenic effect **(Entrican, 2002).** In contrast, IL-10 and TGF-β are beneficial to pregnancy and tend to predominate. Furthermore, damage to chorionic epithelial cells that produce progesterone will alter the hormone balance, leading to an increase in PGE2 and initiating fetal expulsion.

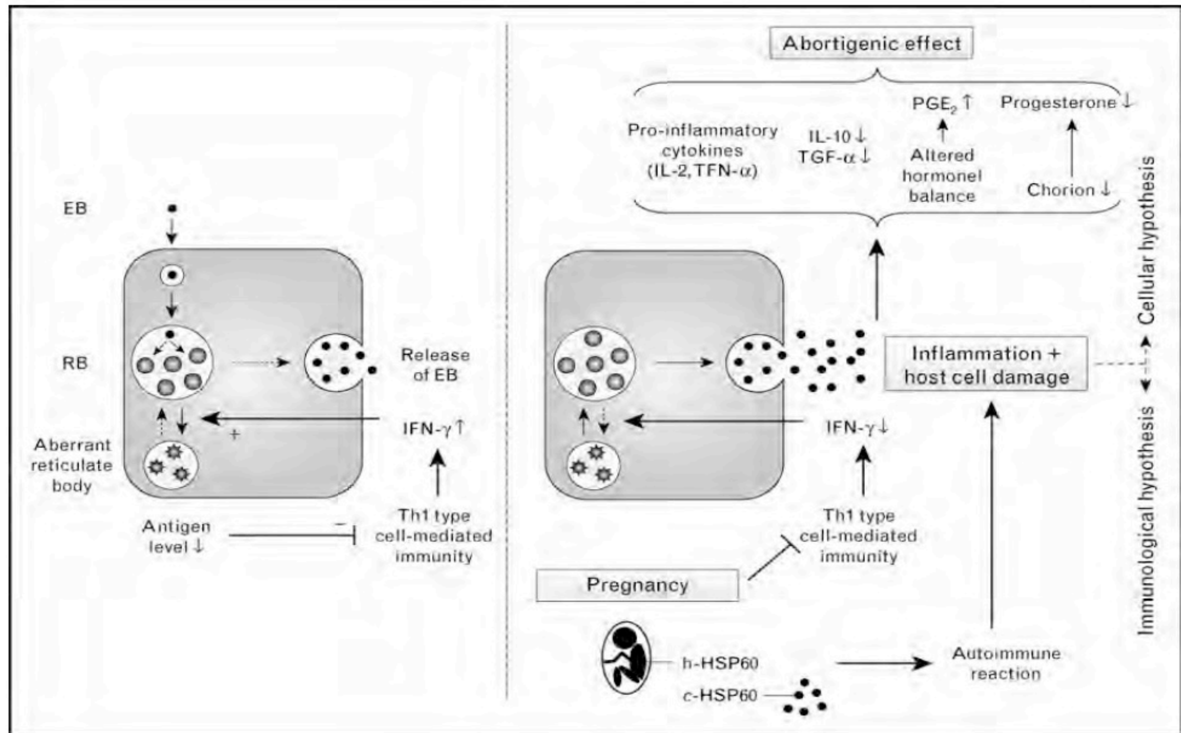



**Fig. 1.10:** Pathogenesis of *Chlamydia*-associated spontaneous abortion (*Baud et al., 2008)*.

### 1.2.14. Association of cyclooxygenases/prostaglandins with spontaneous abortion

Human labour is associated with increased prostaglandin (PG) synthesis within the fetal membranes. The expression of the two isoforms of the central PG synthetic enzyme, cyclooxygenases (COX-1 and COX-2), in human fetal membranes throughout pregnancy, is crucial. COX, also known as PGH synthase, catalyzes the conversion of arachidonate to PGH-2 and is a key enzyme in the regulation of PG formation. COX-1 is constitutively expressed in many tissues **(Garavito *et al.,* 1999).** In sharp contrast, expression of COX-2 can be induced by cytokines/growth factors or inflammatory stimuli. COX-1 plays a crucial role during parturition **(Reese *et al.,* 2000)** while COX-2 is important during ovulation, fertilization, implantation and decidualization **(Lim *et al.,***

**1997).** The role of COX-2 has been elucidated in pregnancy and it has been suggested that the upregulation of COX-2 mediates increased PG synthesis within the foetal membranes at term and much of the increase in COX-2 expression is probably a cause of labour **(Slater *et al.,* 1999).** The majority of COX-2 related studies have focussed on menstruation, preterm birth, foetal death, etc., **(Silver *et al.,* 1995; Smith *et al,* 2007)**.

PGs play an important role in reproduction **(Ni *et al.,* 2002; Dery *et al.,* 2003).** The processes of ovulation and implantation are considered similar to proinflammatory responses hence the involvement of PGs in these processes has long been postulated. The principal site of action for PGs during labour is the myometrium, where they act *via* specific receptors to modulate contractility **(Senior *et al.,* 1993).** Various unique PG receptors have been classified on the basis of their specificity for various agonists and antagonists in both animal and human tissues **(Coleman *et al.,* 1994).** EP receptor has four subtypes (EP1, EP2, EP3 and EP4). EP2, EP4, IP, and DP receptors are termed as "relaxant" receptors. EP1, EP-3 and FP receptors are coupled to phospholipase-C and constitute the "contractile" receptor group **(Smith *et al.,* 1996).**

It has been suggested that an alteration in the PG relaxant or contractile receptors expression could be involved in the maintenance of uterine quiescence during gestation and activate the uterus to contract at the time of parturition for the fetus expulsion **(Brodt-Eppley & Myatt, 1998).** Various studies have shown the expression of COX/ PGs contractile and relaxant receptors in uterine and intrauterine tissues at the time of pregnancy establishment, at term and at parturition **(Smith *et al.,* 2001; Challis *et al.,* 2002)** in a variety of species. During pregnancy, ascending infection into the uterus is followed by local increase of COX-2 activity, and consequently elevated

prostaglandin production. PGF2α has also been considered as the primary candidate present during pregnancy and plays an important role during parturition by increasing contractions in the myometrium **(Fuchs, 1987).** An increase in intrauterine PGF concentrations in pregnant mice treated with LPS has been demonstrated and it was concluded that systemic administration of PGE2 and PGF2α resulted in murine fetal death **(Skarnes & Harper, 1972).** It was also reported that LPS-induced decidual prostanoid production *via* increased COX-2 expression and COX-2 mediated eicosanoid production is probably a key pathophysiologic event in the LPS-mediated fetal death **(Silver *et al.,* 1995).** It was also suggested that two to three-fold increase in uterine and ovarian PG concentrations coincided with the induction of COX-2 **(Gross *et al.,* 2000).**

### 1.2.15. Association of hormonal levels with spontaneous abortion

A well-developed placenta secretes adequate amounts of estrogen and progesterone. Both these hormones are responsible for maintaining the ovum during its early growth period, and if their secretion is inadequate, early abortion may result. The balance between the effects of estrogen and progesterone is critical to maintenance of pregnancy and the onset of labor. During pregnancy, concentration of estrogen increases with progressing gestational age. Estrogen promotes a series of myometrial changes including increased production of PGE2 and PGF2α **(Fuchs *et al.*, 1993).** The physiological function of progesterone is to inhibit smooth muscle contractility, decrease PG formation which help maintain myometrial quiescence and prevent the onset of uterine contractions, and inhibit immune responses like those involved in graft rejection. Serum progesterone has been proposed as a useful test to distinguish a viable pregnancy

from a miscarriage or ectopic pregnancy. Low progesterone values are associated with miscarriages and ectopic pregnancies. In animals, systemic progesterone withdrawal is an essential component in initiation of labour. Spontaneous onset of labour is preceded by a physiologic withdrawal of progesterone activity at the level of uterine receptors **(Madsen *et al.,* 2004).** Progesterone downregulates prostaglandin production, as well as the development of calcium channels and oxytocin receptors both involved in myometrial contraction **(Da Fonseca *et al.,* 2003).** Decreased levels of progesterone and increased level of estrogens affects the PG production leading to spontaneous rupture of the fetal membranes **(Ishihara *et al.,* 1995). (Fig. 1.11)**

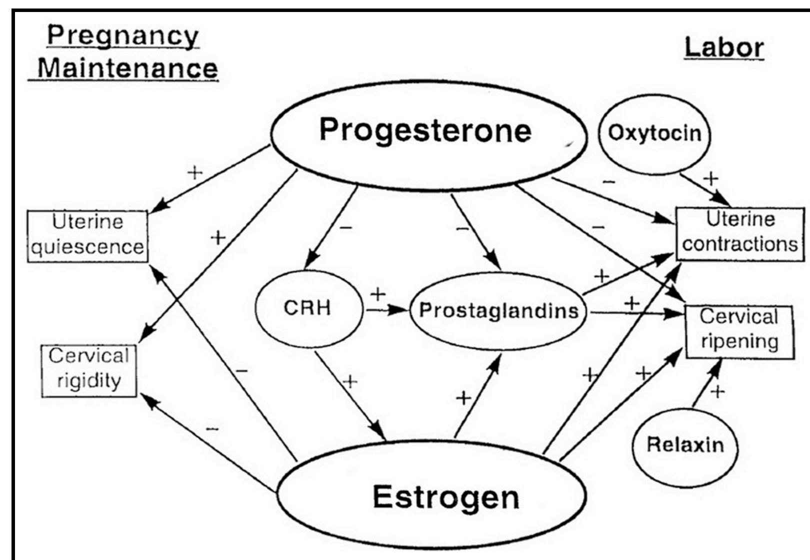



**Fig. 1.11:** Endocrinological control of pregnancy and parturition in women (*Kota et al., 2013).*

The endocrinology of infectious abortion has been the subject of intermittent attention, however, this is restricted largely to animal studies. Studies in mice **(Saksena & Lau, 1973)** and in guinea pigs **(Blatchley *et al*., 1972)** showed that progesterone as well as estrogen was capable of causing synthesis and/ or release of PGs from the uterine

tissue. LPS alters the serum level of progesterone and estrogen during the preimplantation days of pregnancy and elevates the estrogen/ progesterone ratio in the mouse **(Agarwal *et al*., 2010).** The combination of estrogen and progesterone seem to stimulate PG production in ovariectomized animals **(Saksena & Lau, 1973).** Changes in the concentrations of estrogen and progesterone influence the release of PGE2 at parturition **(Olson *et al.,* 1984).** Various studies suggested that decreased endogenous progesterone in the uterus increased uterine PG **(Loose & Stancel, 2006)** and its supplementation has been widely used to prevent miscarriage. The altered ratio of progesterone: estrogen in favour of the latter, upregulates the synthesis of uterine PG and labour **(Challis *et al*., 2000).**

### 1.2.16. Association of cytokines with spontaneous abortion

The immune system within the female genital tract faces the unique challenge of protecting the host against infectious pathogens while being tolerant to the local beneficial microbiota and to an immunologically distinct fetus. Cytokines are immunoregulatory proteins with many functions, ranging from proliferation to differentiation to inhibition. Immunologic cytokines may facilitate or hinder pregnancy, depending on the type of cytokine present, their concentration, and the timing of their secretion relative to gestational age. It has been reported that many miscarriages are caused by abnormal levels of cytokines to control the inflammatory process in the body. Early exposure to proinflammatory cytokines is necessary in stimulating invasion of the blastocyst and the formation of new blood vessels during implantation. However, if the exposure to proinflammatory cytokines is prolonged, it can actually be severely detrimental to the pregnancy and result in a loss of the fetus. Several cytokines can be

detrimental to pregnancy has been established beyond reasonable doubt. Studies suggested that the administration of TNF-α, IFN-γ and IL-2 into pregnant mice causes abortions and low doses of anti-TNFα antibodies reduce resorption rates in a murine abortion model **(Chaouat *et al.,* 1990).** A strong murine anti-parasite response, which includes high placental levels of IFN-γ and TNF-α is accompanied by fetal resorption **(Krishnan *et al.,* 1996).** Furthermore, TNF-α and IFN-γ inhibited outgrowth of human trophoblast cells *in vitro* and stimulated the programed death of human primary villous trophoblast cells **(Yui *et al.,* 1994).**

It was further documented that a significantly higher expression of these cytokines was found in the cultured peripheral blood mononuclear cells of pregnant women with history of recurrent pregnancy loss **(Bates *et al.,* 2002)**. Furthermore, in a study, the expression of IL-6 and IL-8 in cervical mucus was found significantly higher in idiopathic recurrent miscarriage cases who subsequently miscarried than in those who had a live birth **(Hattori *et al.,* 2007)**. IFN-γ also promotes the destruction of *C. trachomatis* but can also promote inflammatory damage and fibrosis **(Rottenberg *et al.,* 2002)**.In mammals, deregulated expression of cytokines and their signaling leads to an absolute or partial failure of implantation and abnormal placental formation **(Guzeloglu-Kayisli *et al.,* 2009).** There are epidemiologic and experimental data indicating that intrauterine infection is a pathological condition that disrupts this privilege **(Makhseed *et al.,* 2003; Peltier 2003).** In response to infection, an abnormal production of proinflammatory cytokines, such as IL-1β, TNF-α, IL-6, and IL-8 were found in the placenta **(El-Shazly *et al.,* 2004)** and chorioamniotic membranes **(Zaga *et al.,* 2004, Zaga-Clavellina *et al.,* 2005),** which may lead to spontaneous abortion**.**

**1.2.17. Association of matrix metalloproteinases & Tissue inhibitors of matrix metalloproteinases with spontaneous abortion**

MMP are a large family of zinc-dependent proteinases capable of degrading native collagen at a physiological pH **(Kahari & Saarialho-Kere, 1999)** and have the ability to break down several proteins of the extracellular matrix (ECM). MMP activity in endometrium has been shown to depend on the phase of the menstrual cycle**.** The gelatinases MMP-2 and MMP-9 are found involved in successful cytotrophoblast invasion in early pregnancy **(Moore *et al.,* 2012).** MMP-2 and MMP-9 are the main mediators of endometrial ECM turnover during menstruation **(Kim *et al.,* 2004).** An alteration in this turnover before implantation has been reported to occur in idiopathic recurrent spontaneous miscarriage (IRSM) **(Jokimaa *et al.,* 2002).** It was further reported that a significant increase in the expression of MMP-9 with no significant changes in MMP-2 in the endometrium of women with IRSM compared with controls **(Skrzypczak *et al.,* 2007).** In contrast, one study found a higher expression of MMP-2 mRNA in the endometrium of women with recurrent miscarriage **(Jokimaa *et al.,* 2002)**. An abnormally elevated expression was observed in human endometrium during the receptive phase in women suffering from infertility **(Tang *et al.,* 2005).** Also, an activity of MMP-2 in implantation window was high and an improper turnover of endometrial ECM was found to be associated with disturbances in implantation and functional bleeding **(Inagaki *et al.,* 2003).**

MMP family members are inhibited by endogenous tissue inhibitors namely tissue inhibitors of matrix metalloproteinases (TIMPs). The balance between MMPs and TIMPs determines the invasiveness of cells, and the homeostasis of the two plays a very

important role in numerous *in vivo* physiological and pathological processes. There have been an increasing number of studies concerning the roles of MMPs and TIMPs in the abortion process. A 25 times higher ratio of MMP-9/TIMP-1 in IRSM suggested the excessive degradation of the ECM which, in turn, affects proper remodelling of the endometrium leading to IRSM **(Banerjee *et al.,* 2013).** MMPs and TIMPs both appear to play some roles in embryo implantation, trophoblast invasion, early placentation, and cervical dilatation and feto-maternal membrane lysis in later gestation **(Goldsmith *et al.,* 2005).**

## 1.3. Gaps in existing research

Despite significant advances in reproductive medicine over the past years, understanding of the mechanisms that determine the establishment of a successful pregnancy is incomplete. As a result, many questions remain unanswered about the optimal management of early pregnancy loss. Previous studies suggest that *C. trachomatis* have been found associated with spontaneous abortions and other adverse pregnancy outcomes but the exact pathway leading to infection induced abortion has not yet studied. Thus in the present work, it is proposed to explore a new potential mechanism for spontaneous abortion in *C. trachomatis* infected patients by hypothesizing a possible role of cyclooxygenase-derived prostaglandin signalling pathway in abortion and its regulation by estrogen/ progesterone hormones in spontaneous aborters infected with *C. trachomatis*. Furthermore, few cytokines (TNF-α, IFN-γ, IL-8, TGF-β1 and TGF-β2) induced by *C. trachomatis* infection, which may trigger this COX/ PG pathway leading to spontaneous abortoions are also undertanken in the study. Additionally, the

expression of key factors responsible for matrix turnover (MMP-2, MMP-9, and their inhibitors TIMP-1/ TIMP-3) is also explored in this study. Studies on the underlying immunomolecular mechanism responsible for spontaneous expulsion in *C. trachomatis* infected spontaneous aborters will enable the development of management/ intervention strategies.

## 1.4. Aims and objectives

The aims and objectives of the study were as follows:

1. To detect *Chlamydia trachomatis* infection in endometrial curettage tissues of aborters
2. To study the expression/ distribution of cyclooxygenases and prostaglandin receptors ($PGE_2$, $PGF_{2\alpha}$, $PGI_2$) alongwith hormone levels in *C. trachomatis* infected spontaneous aborters
3. To ascertain the expression of few proinflammatory cytokines, *viz.*: IFN-γ, TNF-α and IL-8 and multifunctional cytokine TGF-β1/ TGF-β2 in spontaneous aborters infected with *C. trachomatis*
4. To find whether matrix metalloproteinases (MMP-2/ MMP-9) and their inhibitors (TIMP-1/ TIMP-3) are involved in endometrial extracellular matrix turnover in *C. trachomatis*-infected spontaneous aboroters.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Selection and enrollment of patients

**With hospital Ethical Committee permission, a total of 300 aborters** were enrolled from Department of Obstetrics & Gynecology, Safdarjung hospital, New Delhi as follows:

(i) **Group I (Study group): 150 women** undergoing spontaneous abortion by dilation and evacuation at gynecology receiving room (GRR) of the hospital. Group I was further sub-divided in to sporadic spontaneous aborters (SSA; n= 49) and recurrent spontaneous aborters (RSA; n= 101).

(ii) **Group II (Controls): 150 age-matched** healthy pregnant women undergoing medical termination of pregnancy by dilatation and curettage at Family Planning OT of the hospital.

Prior informed written consent was taken from each patient.

## 2.2. Collection of data/ history

Detailed information of age, obstetric/ medical/ gynaecological history including date of last menstrual period, gravidity/ parity/ abortion history and past genital/ urinary tract infection and details about any other medication or treatment were collected in standardized questionnaires from all the enrolled patients.

- **Inclusion criteria**

Women presenting with bleeding per vagina, abdominal pain, passage of clots and experiencing incomplete spontaneous abortion in the first trimester of pregnancy.

- **Exclusion criteria**

Exclusion criteria were as follows:

- Women with local causes of bleeding
- HIV-positivity
- VDRL-positivity (Syphilis infection)
- TORCH positivity (Infection of *Toxoplasma gondii*, *Rubella*, *Cytomegalovirus, Herpes simplex virus*)
- History of recently treated sexually transmitted infection including *Chlamydia trachomatis*
- Genital tuberculosis
- Recent antibiotic therapy
- Any anatomical abnormality

## 2.3. Collection of endometrial curettage tissue/ serum from enrolled patients

Endometrial curettage tissue (ECT) and non-heparinized venous blood samples were collected from all enrolled aborters.

- **Endometrial curettage tissue**

ECT was collected from all the enrolled patients of study group (Group I) and control group (Group II). ECT was evacuated by the gynecologists and transported in PBS on ice to the laboratory. A part of ECT samples were fixed in neutral buffered formalin and embedded in paraffin for histopathological and immunohistochemical

studies. Another part of the ECT was used for DNA extraction and the remaining tissue was stored in RNA later at -80$^{o}$C for RNA isolation and further downstream studies (RT-PCR, Real time PCR).

- **Blood**

5 ml of non-heparinized venous blood was collected in sterilized vials. Serum was separated and stored at -20$^{0}$C in aliquots for further diagnosis of *C. trachomatis.*

## 2.4. Detection of TORCH-associated microorganisms in sera by ELISA

ELISA was performed using the sera of patients (Groups I - II) for detecting the presence of specific IgM antibodies to *Toxoplasma gondi*, Rubella virus, Cytomegalovirus (CMV) and Herpes simplex virus (HSV) by using standard commercial kits (IBL International, Hamburg, Germany) for each microorganism following the manufacturer's guidelines. Briefly, all the specimens and kit reagents were brought to room temperature (18-26 °C) and gently mixed. Before assaying, all samples were diluted 1: 100 with sample diluent. 10 µl sample and 1 ml of sample diluent was mixed into tubes to obtain a 1:100 dilution and thoroughly mixed by vortexing. 100 µl of diluted samples, standards and controls were dispensed into the appropriate wells and incubated for 1 hour at room temperature. Wells were washed 3-4 times with 300µl of washing solution and blotted on absorbance paper or paper towel. 100µl of *Toxoplasma gondii* conjugate was also dispensed to each well and incubated for 1 hour at room temperature and washed wells three times with 300µl washing solution. 100 µl of TMB substrate was dispensed into all wells and incubated for 15 minutes at room temperature. Further, 100µl

of stop solution was added into it and the O.D. was read at 450 nm using ELISA reader within 15 min.

**2.5. Molecular detection of Chlamydia trachomatis in endometrial curettage tissue**

**2.5.1. Isolation of DNA**

For extracting DNA, Wizard Genomic DNA Isolation kit (Promega, U.S.A.) was used and DNA was isolated as per the manufacturer's instructions. Briefly, the ECT was homogenized in liquid nitrogen and treated with lysis buffer and centrifuged. The lysate was thereafter treated with protein precipitation solution. Finally the DNA was precipitated by isopropanol and stored at -20$^0$C.

**2.5.2. Qualitative and quantitative analysis of extracted DNA**

Qualitative and quantitative analysis of extracted DNA was performed before the PCR assay. Quality of DNA was checked for any shearing by running DNA samples on 0.8% agarose gel and and had a bright illuminated band on transilluminator (Fig. 2.1). DNA samples were quantified on a nano-drop spectrophotometer (Fermentas-Thermo Fisher Scientific, Waltham, USA) at 260 nm. 260:280 ratio was ~ 1.8 for all the DNA samples.

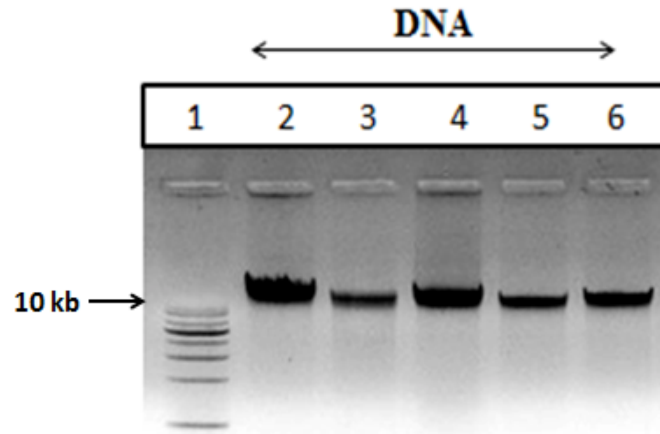



**Fig: 2.1:** Agarose gel electrophoresis products visualized under Gel documentation system; Qualitative analysis of purified DNA in ECT: Lane 1- 1 kb DNA Ladder; Lane 2-6 - DNA samples from different patients

### 2.5.3. Selection of primers for performing PCR assays for *Chlamydia trachomatis* detection

For detection of *C. trachomatis* in ECT, *C. trachomatis* MOMP gene, extra-chromosomal endogenous plasmid gene and 16S rRNA gene primers were used. The sequence of the primer pairs for plasmid gene **(Joyee *et al.,* 2003)**, MOMP gene **(Palmer *et al.,* 1991) and 16S rRNA gene (Wilkinson *et al.,* 1998)** were selected from the published literature and commercially synthesized from *m/s Biolinkk, New Delhi, India.* List of primers used is given in the **Table 2.1**

**Table 2.1:** Primers for molecular diagnosis of *Chlamydia trachomatis* in the ECT

| Gene name | Primer Sequence: 5'-3' | Amplicon size (bp) | Reference |
|---|---|---|---|
| **MOMP** | F: TAT ACA AAA ATG GCT CTC TGC TTT AT<br>R: CCC ATT TGG AAT TCT TTA TTC ACA TC | 537 bp | *Palmer et al., 1991* |
| **Plasmid gene** | F: GGACAAATCGTATCTCGG<br>R: GAAACCAACTCTACG CTG | 517 bp | *Barlow et al., 2001* |
| **Plasmid gene** | F: CTA GGC GTT TGT ACT CCG TCA<br>R: TCC TCA GGA GTT TAT GCA CT | 200 bp | *Joyee et al., 2003* |
| **16S rRNA Gene** | F: TGAGGCATGCAAGTCGAAC<br>R: TTACTCGGATGCCCAAATATC | 150 bp | *Wilkinson et al., 1998* |
| **Beta globin** | F: CAA CTT CAT CCA CGT TCA CC<br>R: GAA GAG CCA AGG ACA GGT AC | 268 bp | *Nikkari et al., 1994* |

Abbreviation: MOMP- Major outer membrane protein of *Chlamydia trachomatis*

**Nucleic acid amplification tests**

PCR assays were performed with β-globin, *C. trachomatis* MOMP, plasmid, and 16S rRNA gene using PCR thermal cycler *(Applied Bio System, USA)*. The PCR products were detected by agarose gel electrophoresis of the amplified products in 1.2% agarose gel. A 100 bp DNA ladder *(Bangalore Genei, Bangalore, India)* was used as marker for the DNA size standard. The DNA was visualized on an UV-Transilluminator or on gel documentation unit.

### 2.5.4. Polymerase chain reaction with β-globin gene

Each DNA specimen was subjected to internal control human β-globin gene specific PCR to check for the suitability of the processed DNA sample and to check any other contamination or shearing in DNA for further PCR purposes. PCR assay was performed in ECT samples for the diagnosis of *C. trachomatis* infection. For this, amplification reaction was set up in 25µl reaction volume using PCR Thermal Cycler (**Table 2.2).** The genomic DNA was initially denatured at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds while primer annealing at 55°C for 1 minute for *C. trachomatis* plasmid gene and extension at 72°C for 45 seconds. The cycle number was optimized to 35 cycles. Final extension was carried out at 72°C for 10 minutes to obtain a 268 bp for β-globin gene **(Nikkari *et al*., 1994)**. The amplified product was confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV transilluminator **(Fig. 2.2).**

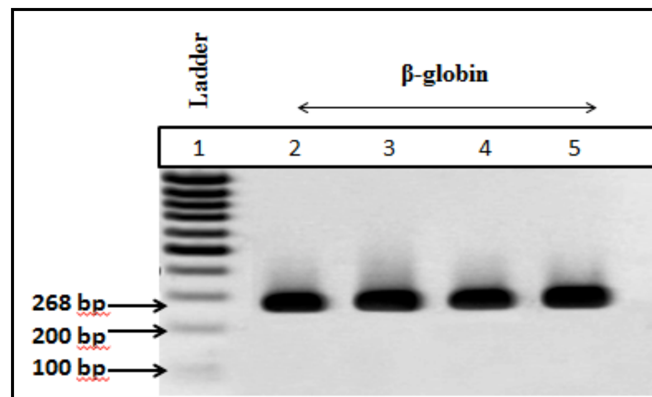



**Fig. 2.2:** Agarose gel electrophoresis products visualized under UV-Transilluminator; Lanes 2 - 5 shows DNA of different clinical specimens (ECT) with β-globin gene (268bp)

### 2.5.5. Detection of *Chlamydia trachomatis* endogenous plasmid in endometrial curettage tissue by PCR assay

PCR assay was performed in ECT samples for the diagnosis of *C. trachomatis* infection. For this, amplification reaction was set up in 25µl reaction volume using PCR Thermal Cycler (**Table 2.2, Fig. 2.3).** Final extension was carried out to obtain a 200 bp product for *C. trachomatis* plasmid gene **(Joyee *et al.*, 2003).** The amplified product was confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV transilluminator.

**Table 2.2:** Reaction mixture (25 µl) for PCR

| | |
|---|---|
| 10x Buffer KCl (50 mM) & Tris-HCl (10 mM) (pH-8.4) | 2.5 µl |
| $MgCl_2$ (25 mM) | 1.5 µl |
| dNTPs (0.2 mM) | 0.5 µl |
| Forward primer (10 picomole/µl) | 0.5 µl |
| Reverse primer (10 picomole/µl) | 0.5 µl |
| Taq DNA polymerase (500 Unit) | 0.4Unit |
| Template DNA | 5 µl |
| Distilled water (molecular grade) | 14.5 µl |

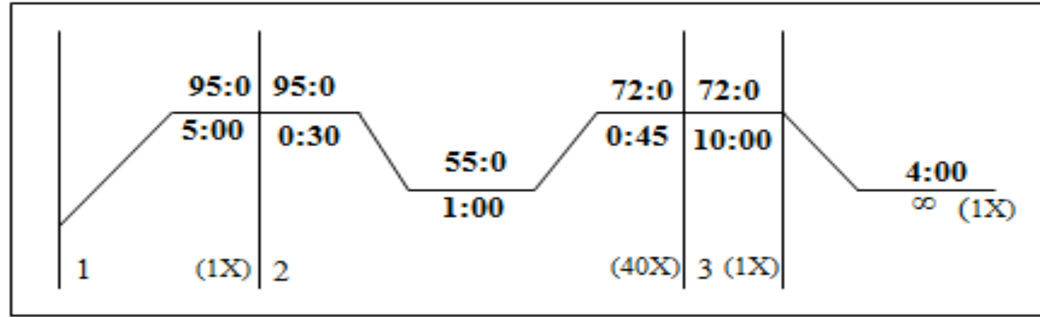



**Fig 2.3:** Conditions for PCR with *Chlamydia trachomatis* plasmid gene

### 2.5.6. Detection of *Chlamydia trachomatis* major outer membrane protein (MOMP) by PCR assay

Polymerase Chain Reaction (PCR) assay was performed in endometrial curettage tissue (ECT) samples for the diagnosis of *C. trachomatis* infection. For this, amplification reaction was set up in 25µl reaction volume using PCR Thermal Cycler **(Table 2.2, Fig. 2.4).** Final extension was carried out at 72$^{o}$C to obtain a 537 bp product for *C. trachomatis* plasmid gene **(Palmer et al., 1991).** The amplified product was confirmed by electrophoresis on a 2% agarose gel.

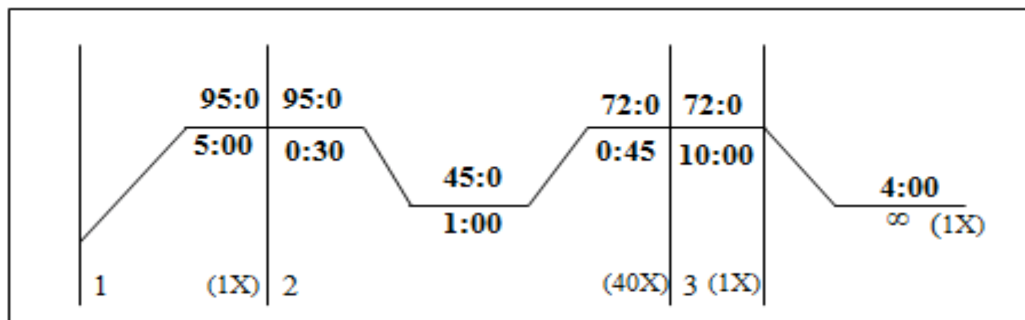



**Fig 2.4:** Conditions for PCR with *Chlamydia trachomatis* MOMP specific gene

### 2.5.7. Detection of *Chlamydia trachomatis* 16S rRNA gene by PCR assay

Polymerase Chain Reaction (PCR) assay was performed in endometrial curettage tissue (ECT) samples for the diagnosis of *C. trachomatis* infection. For this, amplification reaction was set up in 25µl reaction volume using PCR Thermal Cycler **(Table 2.2, Fig. 2.4).** Initially, DNA was denatured at 95$^{o}$C for 5 minutes and thereafter, for 30 seconds per cycle while annealing was done at 48$^{o}$C for 1 minute. Extension was done at 45 seconds and the final extension carried out to obtain a 150 bp product for *C. trachomatis* plasmid gene. The amplified product was confirmed by electrophoresis on a 2% agarose gel. The PCR was subjected to 40 cycles.

### 2.5.8. Sequencing of PCR products

After confirmation of *C. trachomatis* infection in SA by MOMP/ plasmid gene, the PCR products were subjected to sequence analysis. A commercial QIAquick Gel Extraction kit was used to extract DNA from the agarose gel. DNA fragment was excised from the gel with a clean, sharp scalpel and the gel slice was collected in a colourless tube after weighing. Purified product was used for DNA sequencing in an Automated Sequencer *(ABI PRISM 3130 XL, Foster City, CA)*. Resulted sequences were blasted in NCBI through non-redundant blast against *C. trachomatis* genome database.

### 2.5.9. Detection of *Chlamydia trachomatis* in ECT by quantitative real time

Quantitative Real Time PCR (q-PCR) assay was performed on Step-one plus Real-time PCR system (*Applied Biosystems, USA*) to detect the chlamydial load in conventional PCR positive ECT samples. To quantitate the copy number of *C. trachomatis* in ECT, firstly, serial dilutions (10000 µl, 1000µl, 100µl, 10µl, 0.1 µl) of *C.*

*trachomatis* DNA positive control *(Vircell microbiologist, Spain)* were prepared as per manufacturer's instructions. Briefly, 10µl of SYBR green master mix, 1µl of plasmid gene (200 bp) forward primer and 1µl of reverse primer **(Joyee *et al.,* 2003)**, 5µl of Vircell *C. trachomatis* diluted DNA of each concentration and 3µl of nuclease free water were mixed to make up a final volume of 20µl. The reactions were set in duplicates for each concentration and standard curve was drawn. By using this standard curve, the chlamydial load was calculated in each ECT sample. The Threshold Cycle (Ct) value was calculated as an average Ct target genes for each sample **(Fig. 2.5).**

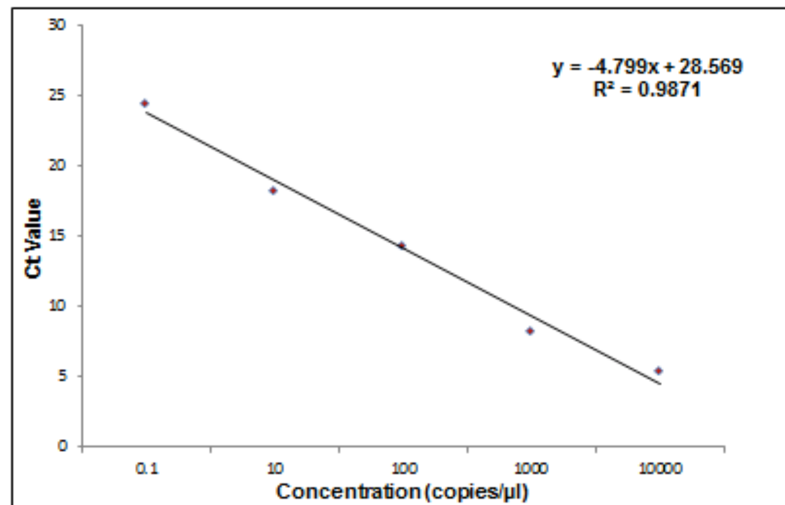



**Fig. 2.5:** Standard curve for *Chlamydia trachomatis* Positive Control

## 2.6. Semi-quantitative analysis of mRNA expression of various genes by reverse transcription-PCR

### 2.6.1. Isolation of RNA from endometrial curettage tissue

The total RNA was isolated from the ECT by using Trizol reagent *(Invitrogen, USA)* according to the manufacturer's instructions. Briefly, disruption of the tissue sample was done by taking 20 - 30 mg of the ECT and thereafter, homogenization was done with 1 ml Trizol reagent with 200µl chloroform. The lysate was centrifuged at 40$^{o}$C and 75% ethanol was added to the supernatant. The tube was re-centrifuged and the pellet air-dried. 30µl of RNase-free water was added and the resulting RNA was stored at -80$^{o}$C. The purity was checked by running the samples on 1% agarose gel **(Fig. 2.6)** and by taking the nanodrop OD. A 260/ 280 ratio, *i.e.* 1.9 - 2.0 was considered as optimum.

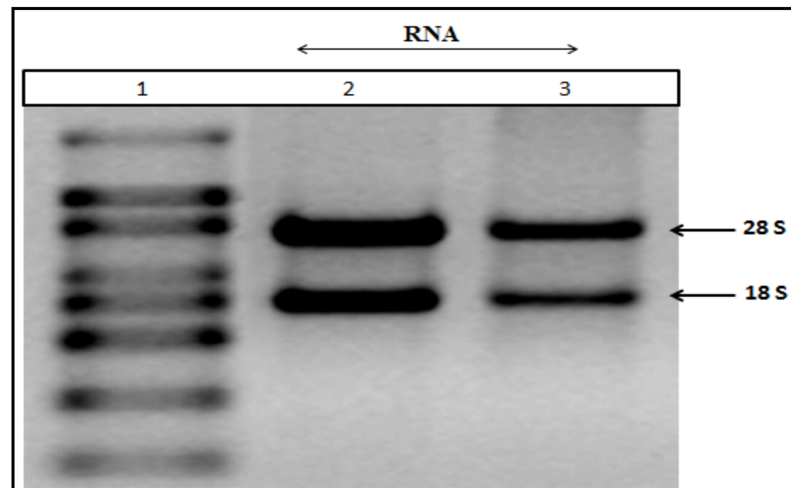



**Fig. 2.6:** Agarose gel electrophoresis products visualized under UV-transilluminator; **Lane 1** shows the RNA ladder. Lanes 2- 3 shows RNA in endometrial curettage tissue.

### 2.6.2. Synthesis of cDNA from RNA

For cDNA synthesis, total RNA (1.5 μg) was used to synthesize first strand cDNA using High Capacity cDNA Reverse Transcription Kit with the treatment of RNase Inhibitor *(Applied Biosystems, USA)* by One-Step Reverse Transcription-PCR as per the manufacturer's instructions briefly as summerized in **Table 2.3** and cDNA Synthesis was performed on PCR Thermal Cycler. The PCR conditions were as follows; initial denaturation for 10 minutes at $94^o$ C, annealing at $50^o$-$68^o$ C for 120 minutes, extension for 5 minutes at $85^o$ C and then $4^o$ C for α time.

**Table 2.3:** Reaction Components for cDNA conversion

| | |
|---|---|
| 10x RT Buffer | 10 μl |
| 25x dNTP Mix (100 mM) | 2 μl |
| 10x RT Random primers | 2 μl |
| RNase inhibitor | 1 μl |
| Template RNA | 1.5 μg |
| Nuclease-free water | *Variable* |

### 2.6.3. Qualitative expression of cyclooxygenases (COX-1 and COX-2) by reverse transcription-PCR

The cDNA expression of COX-1 and COX-2 in the ECT was performed with the constitutively expressed housekeeping gene Beta-actin (*β-actin)* for normalization (**Table 2.4)**. Abundance of receptor mRNA was expressed relative to β-actin mRNA. PCR was

performed in 25µl reaction volume using DNA Thermal Cycler *(Applied Biosystems, USA)* as given in **Table 2.5.** The COX-1 and COX-2 genes were amplified on annealing temperatures 62$^{o}$C and 57$^{o}$C, respectively **(Fig. 2.7, 2.8).** The amplified products were confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV Transilluminator and photographed.

**Table 2.4: Primer sequences for reverse transcription PCR**

| Gene name | Primer Sequence: 5'-3' | Amplicon size (bp) | Reference |
|---|---|---|---|
| β- Actin | F: TGT GAT GGT GGG TAT GGG TC<br>R: ACACGCAGCTCATTGTA | 162 | *Jasper et al., (2006)* |
| COX-1 | F: CGCCTGGTACTCACAGTC<br>R: CATAAATGTGGCCGAGGTCTA | 304 | *Slater et al., (1999)* |
| COX-2 | F: TTCAAATGAGAGATTGTGGAAAATTGCT<br>R: AGATCATCTCTGCCTGAGTATCTT | 305 | *Slater et al., (1999)* |
| EP-1 | F: AGGCACTGCTTGCTGGCCTCTT<br>R: TGGCCCACCATCTCCAC | 295 | *Leonhardt et al., (2003)* |
| EP-2 | F: TGCTGGACTATGGGCAGTACGTCC<br>R: AAGGTGATGGTCATGATAGCCAGG | 304 | *Leonhardt et al., (2003)* |
| EP-3 | F: ACTGGTATGCGAGCCACATGA<br>R: CATAAGCTGAATGGCCGTCTC | 368 | *Leonhardt et al., (2003)* |
| EP-4 | F: TCCGCATGCACCGCCAGTTCAT<br>R: TCGGATGGCCTGCAAATCTGG | 317 | *Leonhardt et al., (2003)* |
| FP | F: TGGAAATGGTAATCCAGCTCCTGG<br>R: CATGCACTCCACAGCATTGACTGG | 246 | *Leonhardt et al., (2003)* |
| IP | F: TTCGTGCAGGAACCTCACCTACG<br>R: TCCAGCTGCGCGTAGAGGTA | 384 | *Leonhardt et al., (2003)* |

**Table 2.5:** Reaction mixture (25 µl) for reverse transcription-PCR

| | |
|---|---|
| 10x Buffer KCl (50 mM) & Tris-HCl (10 mM) (pH-8.4) | 2.5 µl |
| $MgCl_2$ (2 mM) | 1.5 µl |
| dNTPs (0.2 mM) | 0.5 µl |
| Forward primer (10 picomole/µl) | 0.5 µl |
| Reverse primer (10 picomole/µl) | 0.5 µl |
| Taq DNA polymerase (500 Unit) | 0.4 µl |
| cDNA | 4 µl |
| Nuclease free water | 12.5 µl |

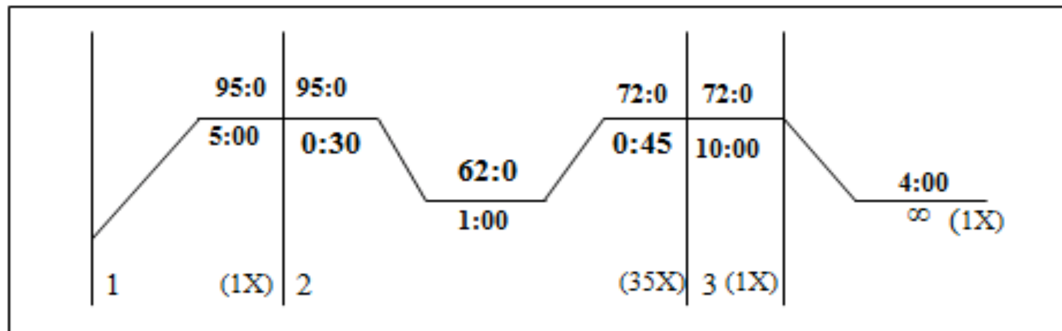



**Fig. 2.7:** Conditions for reverse transcription-PCR with COX-1 gene

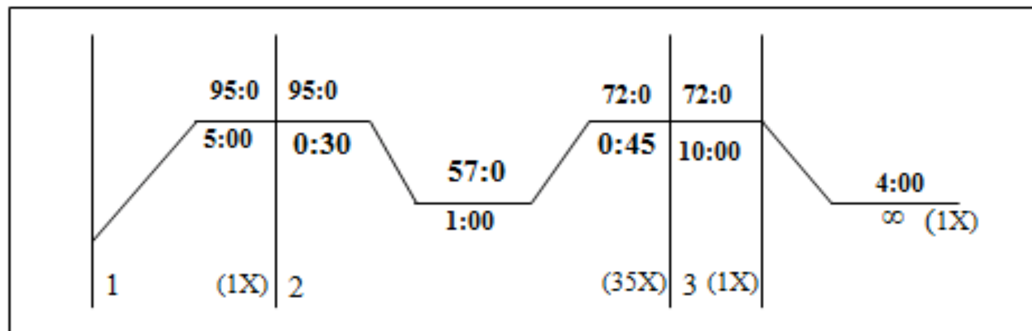



**Fig. 2.8:** Conditions for reverse transcription-PCR with COX-2 gene.

### 2.6.4. Qualitative expression of prostaglandin receptors (*viz.*; EP-1, EP-2, EP-3, EP-4, FP and IP) by reverse transcription-PCR

The cDNA expression of PGE2, PGF2α and PGI2 receptors (*viz.*; EP-1, EP-2, EP-3, EP-4, FP and IP) in ECT was performed with the constitutively expressed housekeeping gene Beta-actin (*β-actin)* for normalization **(Table 2.4)**. PCR was performed in 25µl reaction volume using DNA Thermal Cycler **(Table 2.5).** The EP-1, EP-2, EP-3, EP-4, FP and IP receptor genes were amplified on annealing temperatures of 57°C, 60°C, 55°C, 60°C 56°C and 56°C respectively **(Fig. 2.9- 2.12).**

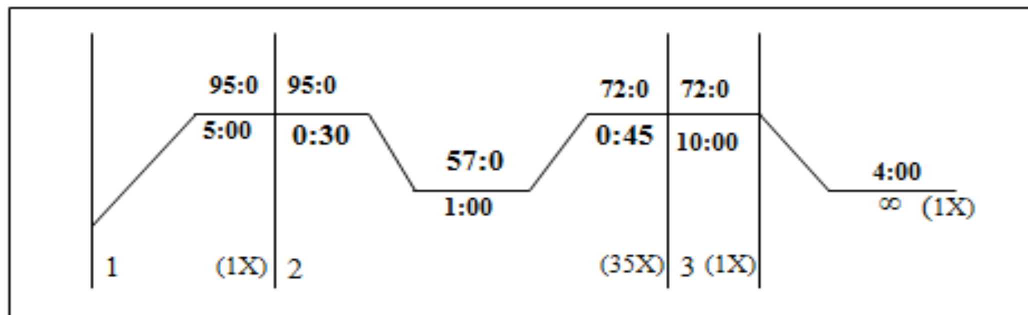



**Fig. 2.9:** Conditions for reverse transcription-PCR with EP-1 gene

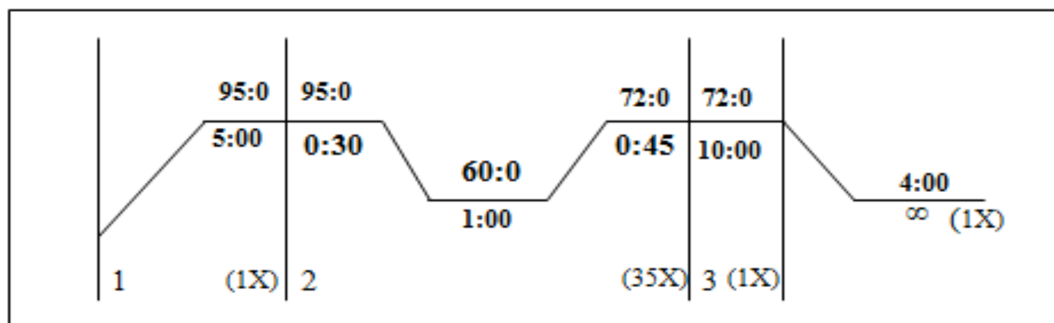



**Fig. 2.10:** Conditions for reverse transcription -PCR with EP-2 and EP-4 gene

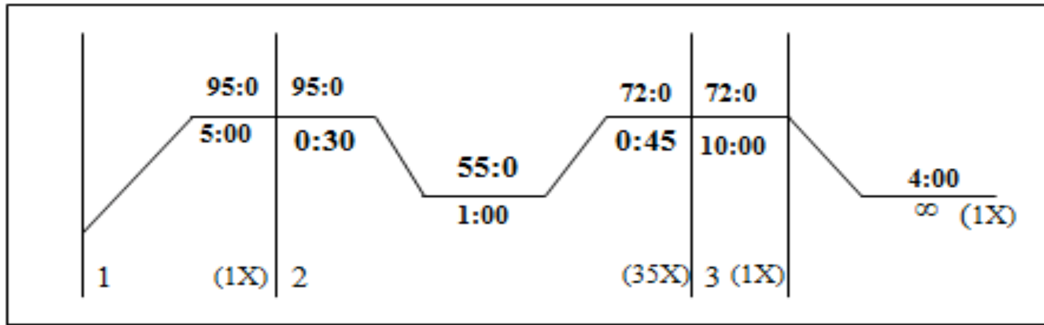



**Fig. 2.11:** Conditions for reverse transcription-PCR with EP-3 gene

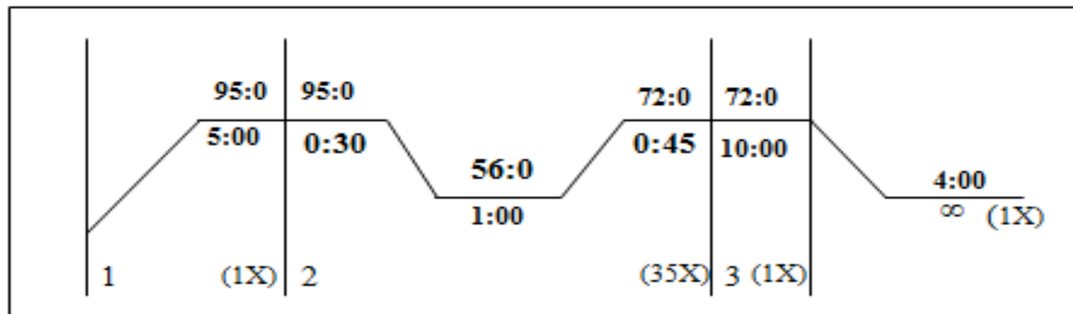



**Fig. 2.12:** Conditions for reverse transcription-PCR with FP and IP gene

### 2.6.5. Qualitative expression of proinflammatory cytokines (*viz.;* TNF-α, IFN-γ and IL-8) and multifunctional tissue growth factors ($TGF\beta_{1/}$ $TGF\beta_{2)}$ by reverse transcription-PCR

RT-PCR for proinflammatory cytokine genes in the ECT was performed in 25µl reaction volume using DNA Thermal Cycler *(Applied Biosystems, USA)*. Housekeeping gene Beta-actin (*β-actin)* was used as an endogenous control for the reactions **(Table 2.5, 2.6; Fig. 2.13).**

**Table 2.6: Primer sequences for cytokines for reverse transcription PCR**

| Gene name | Primer Sequence: 5'-3' | Amplicon size (bp) | Reference |
|---|---|---|---|
| TNF-α | F: GCC CGA CTA TCT CGA CTT TGC<br>R: GGA GGC GTT TGG GAA GGT T | 96 | *Jasper et al., 2006* |
| IFN-γ | F: GAA ACG AGA TGA CTT CGA AAA GCT<br>R: TGT ATT GCT TTG CGT TGG ACA T | 72 | *Jasper et al., 2006* |
| IL-8 | F: ATGACTTCCAAGCTGGCCGTGGCT<br>R: TCTCAGCCCTCTTCAAAAACTTCTC | 289 | *Cheng el al., 2008* |
| TGF-β1 | F: AAA TTG AGG GCT TTC GCC TTA<br>R: TGA ACC CGT TGA TGT CCA CTT | 81 | *Jasper et al., 2006* |
| TGF-β2 | F: CAG TGG GAA GAC CCC ACA TC<br>R: GCC GGT TGG TCT GTT GTG A | 74 | *Jasper et al., 2006* |

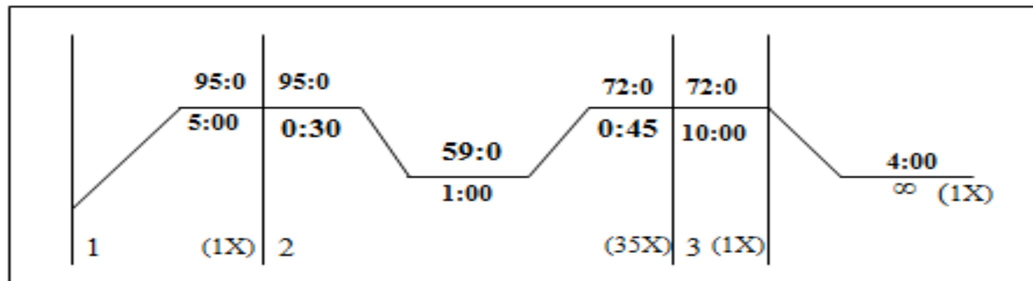



**Fig. 2.13:** Conditions for reverse transcription-PCR with cytokine genes

### 2.6.6. Qualitative expression of matrix metalloproteinase (*viz.*; MMP-2 and MMP-9) and tissue inhibitors of matrix metalloproteinase (*viz.*; TIMP-1, TIMP-3) by reverse transcription PCR

RT- PCR for MMP-2/ MMP-9 and TIMP-1/ TIMP-3 genes was performed in the ECT samples to study the qualitative expression of these genes. 25µl reaction volume

was amplified using DNA Thermal Cycler *(Applied Biosystems, USA)*. Housekeeping gene Beta-actin (*β-actin)* was used as an endogenous control for the reactions **(Table 2.5, 2.7 Fig. 2.14).** The amplified products were confirmed by electrophoresis on a 2% agarose gel, visualized under UV Transilluminator and photographed.

**Table 2.7: Primer sequences for MMPs/ TIMPs for reverse transcription PCR**

| Gene name | Primer Sequence: 5'-3' | Amplicon size (bp) | Reference |
|---|---|---|---|
| MMP-2 | F: AAGTATGGCTTCTGCCCTGA<br>R: ATTTGTTGCCCAGGAAAGTG | 270 bp | *Skrzypczak et al., (2007)* |
| MMP-9 | F: TCTTCCCTGGAGACCTGAGA<br>R: ATTTCGACTCTCCACGCATC | 300 bp | *Jiang et al., (2015)* |
| TIMP-1 | F: TGACATCCGGTTCGTCTACA<br>R: TGCAGTTTTCCAGCAATGAG | 313 bp | *Skrzypczak et al., (2007)* |
| TIMP-3 | F: TCCCTTGGACACTAACTCTTCC<br>R: CCTCCCTCACTCTTACATG | 593 bp | *Jiang &Qi (2015)* |

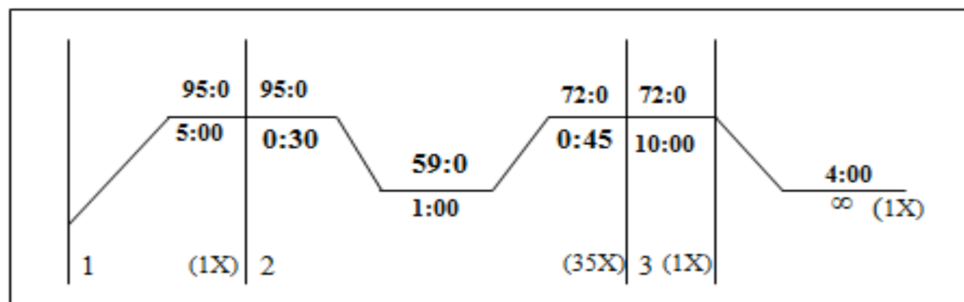



**Fig. 2.14:** Conditions for RT-PCR with MMP-2/ MMP-9, TIMP-1/ TIMP-3 genes

## 2.7. Quantitative analysis of mRNA expression of various genes by real time PCR (q-PCR)

Quantitative Real Time PCR (q-PCR) assay was performed on 7000 Real-time PCR system (*Applied Biosystems, USA*) for studying the quantitative expression of various genes, *viz.:* COX-1/ COX-2, PGE2, PGF2α and PGI2 receptors (EP-1, EP-2, EP-3, EP-4, FP, IP), cytokines (TGF$\beta_{2,}$ TNF-α, IFN-γ, IL-8, TGF-β1, TGF-β2 genes) and MMP-2/ MMP-9 and TIMP-1/ TIMP-3 genes in the ECT.

### 2.7.1. Quantitative expression of cyclooxygenases (COX-1/ COX-2), prostaglandin receptors (*viz.*; EP-1, EP-2, EP-3, EP-4, FP and IP) and cytokines (*viz.;* TNF-α, IFN-γ and IL-8) and TGF-β1/ TGF-β2

The expression of various genes in the ECT was determined by using Taqman real time PCR. 1.5 µg/ µl concentration of c-DNA was used for *q*-PCR. The reaction mix was prepared as given in **Table 2.8.** Reaction mix was loaded into the real time optical PCR tubes (*Applied Biosystems, USA*). All the reactions were set in duplicates. The assay was standardized for the internal control gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin (β-actin) in the ECT samples and those showing consistency with GAPDH, were selected for real time PCR studies. The Threshold Cycle (Ct) value was calculated as average Ct target genes for each sample by using SDS software and the relative quantification was used to measure gene expression by relating the PCR signal.

**Table 2.8:** Reaction mixture (20μl) for Taqman q-PCR

| | |
|---|---|
| 2X Taqman master mix | 10μl |
| Probe (Assay on Demand) | 1μl |
| c-DNA | 4μl |
| Nuclease free water | 5μl |

### 2.7.2. Quantitative expression of matrix metalloproteinase (*viz*.; MMP-2 and MMP-9) and tissue inhibitors of matrix metalloproteinase (*viz*.; TIMP-1, TIMP-3) by q-PCR

Quantitative Real Time PCR (q-PCR) assay was performed for studying the quantitative expression of matrix metalloproteinase (*viz*.; MMP-2 and MMP-9) and tissue inhibitors of matrix metalloproteinase (*viz*.; TIMP-1, TIMP-3) in the ECT by using SYBR green based chemistry. 10μl of Power SYBR green PCR master mix (*Applied Biosystems, USA*), 1μl of forward primer (*Biolinkk, New Delhi, India*), 1μl of reverse primer *(Biolinkk, New Delhi, India)*, 5μl of c-DNA (1.5 μg/μl) and 3μl of nuclease free water *(Ambion, Thermo fisher scientific, USA*) were mixed to make up a final volume of 20μl. Finally the reaction mix was loaded into the real time optical PCR tubes (*Applied Biosystems, USA*). All the reactions were set in duplicates. The assay was standardized for the internal control gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin (β-actin) in the ECT samples and those showing consistency with GAPDH and β-actin were selected for real time PCR studies. The Threshold Cycle (Ct) value was

calculated as average Ct target genes for each sample by using SDS software and the relative quantification was used to measure gene expression by relating the PCR signal.

**2.8. Estimation of Estrogen in the serum of aborters**

Serum estrogen concentration was determined by a commercial estradiol ELISA kit (*Cayman, USA*) as per manufacturer's guidelines. Briefly, 100 µl of ELISA buffer was added to the non-specific binding wells followed by the addition 50 µl of standard dilutions to the dilution wells. After the subsequent addition of 50 µl samples per well, each sample was assayed at a minimum of two dilutions and each dilution was assayed in duplicate. Thereafter, 50 µl of estradiol AChE tracer, followed by 50 µl of estradiol ELISA antiserum was added to each well except the non-specific binding and blank wells followed by washing, addition of 200 µl of Ellman's reagent and incubation at room temperature. Finally the plate was read at wavelength between 405 - 420 nm.

**2.9. Estimation of Progesterone in the serum of aborters**

Serum progesterone concentration was determined by a commercial progesterone EIA kit (*Cayman, USA*) as per manufacturer's guidelines. Briefly, 100 µl of EIA buffer was added to the non-specific binding wells followed by the addition of 50 µl of EIA buffer and 50 µl of standard dilutions to the dilution wells. After the subsequent addition of 50 µl samples per well, each sample was assayed at a minimum of two dilutions and each dilution was assayed in duplicate. Thereafter, 50 µl of progesterone AChE tracer, followed by 50 µl of antiserum was added to each well followed by incubation, washing, addition of Ellman's reagent and incubation at room temperature. Finally the plate was read at wavelength between 405 - 420 nm.

### 2.10. Statistical analysis

Statistical analysis was performed by using GraphPad Prism software version 5.0 *(GraphPad Software, Inc., San Diego, USA)* for different variables. Patients from Group I, Group II and Group III, Group IV were compared by Fisher's exact test. *'p'* value for different variables was calculated by using Student's t test and non-parametric Mann-Whitney U test. One-way ANOVA was also used to calculate the level of significance within the groups. A *'p'* value less than or equal to 0.05 was considered to be significant. Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were also calculated to evaluate the diagnostic efficacy. Mean and standard deviations were derived from the numerical data using column statistics.

**2.10. List of kits used:**

| Name of Kit | Name of Manufacturer |
|---|---|
| Wizard Genomic DNA Isolation kit | Promega, Wiscounsin, USA |
| High Capacity cDNA Reverse Transcription Kit | Applied Biosystems, USA |
| *Toxoplasma gondi* IgM ELISA kit | IBL International, Hamburg, Germany |
| Rubella IgM ELISA kit | IBL International, Hamburg, Germany |
| Cytomegalovirus IgM ELISA kit | IBL International, Hamburg, Germany |
| Herpes simplex virus IgM ELISA kit | IBL International, Hamburg, Germany |

**List of reagents used:**

| Name of Reagent | Name of Manufacturer |
|---|---|
| $MgCl_2$ (25 mM) | Fermentas - Thermo Fisher Scientific, Waltham, USA |
| 10x Buffer KCl (50 mM) Tris-HCl (10 mM) (pH-8.4) | Fermentas - Thermo Fisher Scientific, Waltham, USA |
| dNTPs (2mM) | Fermentas - Thermo Fisher Scientific, Waltham, USA |
| Taq DNA Polymerase | Fermentas - Thermo Fisher Scientific, Waltham, USA |
| Distilled water (molecular grade) | Ambion - Thermo Fisher Scientific, Waltham, USA |
| Oligonucleotides | Biolinkk, New Delhi, India |
| Agarose | Bangalore Genei, Bangalore, India |
| Ethidium bromide | Bangalore Genei, Bangalore, India |

| | |
|---|---|
| 100 bp DNA Ladder | Bangalore Genei, Bangalore, India |
| 50 bp DNA Ladder | Bangalore Genei, Bangalore, India |
| Trizol reagent | Invitrogen - Thermo Fisher Scientific, Waltham, USA |
| RNAase Inhibitor | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| COX-1 Probe | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| COX-2 Probe | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| EP-1, EP-2, EP-3, EP-4, FP, IP Probes | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| GAPDH probe | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| Taqman Real time PCR master mix | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| Power SYBR green PCR master mix | Applied Biosystems, Thermo Fisher Scientific, Waltham, USA |
| AMPLIRUN *Chlamydia trachomatis* DNA Control | Vircell Microbiologists, Granada, Spain |

## 2.11. Preparation of reagents:

**Ethidium Bromide (10 mg/ml)**

10 mg of ethidium bromide was dissolved in 1 ml dd$H_2O$, stored in an opaque bottle.

**DEPC water**

0.1% diethylpyrocarbonate was added to 1 liter $ddH_2O$ in a fume hood and mixed well. After incubating it for 1 hr at 37°C it was autoclaved.

**Phosphate Buffer Saline (PBS)**

8 gm of NaCl, 2 gm of KCl, 1.44 gm of $Na_2HPO_4$ and 0.2 gm of $KH_2PO_4$ were dissolved in 800 ml of distilled water. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15 lb/sq.in for 20 min. and stored at room temperature (10mM, pH 7.4).

**10 X TAE buffer (Tris acetate, EDTA)**

4.84 gm of 40mM Tris base in 80 ml of distilled water was dissolved and 1.2 ml of 20mM glacial acetic acid and 2 ml of 0.5M EDTA, pH 8.0 were added. Final volume was made up to 100 ml.

**Dilution of Oligonucleotides**

Lyophilized primers were reconstituted in UV treated nuclease free water according to manufacturer's guidelines.

**Precautions**

During each assay, precautions were taken to avoid cross-contamination. For PCR, RT-PCR and q-PCR, each sample preparation was done in an isolated laminar flow and Positive and negative controls were used during PCR assay to rule out cross contamination. During DNA and RNA isolation procedures, gloves were changed at each

step to avoid contamination and the surface was cleaned carefully by using 70 % alcohol and RNA zap. Sera were prepared from aseptically collected samples using standard techniques. Heat- inactivated sera was not used as growth factor and amino acid get affected. The lipemic, turbidor contaminated sera was not used as the particulate material and precipitates in sera might give erroneous results. Such specimens were clarified by centrifugation or filtration prior to being assayed.

# CHAPTER 3

# FREQUENCY OF *CHLAMYDIA TRACHOMATIS* INFECTION IN ENDOMETRIAL CURETTAGE TISSUES OF ABORTERS

## 3.1. Introduction

*Chlamydia trachomatis* is the causative agent of sexually transmitted infection in humans and is a wide spread public health concern because of its high prevalence. Worldwide, an estimated 131million sexually transmitted *C. trachomatis* infections occur each year **(Newman *et al.,* 2015).** It is a true parasite with an extracellular and intracellular phase of its life cycle, responsible for the chronic and asymptomatic course of infection. The wide spread of these infections and their chronic oligosymptomatic or asymptomatic course pose a great clinical and epidemiological problem. These "silent infections" may lead to dangerous, frequently irreversible complications both in women and men whose treatment is extremely expensive **(Zdrodowska-Stefanow & Ostaszewska, 2000).** Its ability to colonize a host and inflict significant tissue damage is thought to be responsible for a large number of cases of infertility and ectopic pregnancies. Chlamydial infection has been linked to urethritis, proctitis, epididymitis, Bartholin's gland abscess, cervicitis, endometritis, salpingitis, perihepatitis, choriaoamnionitis, premature rupture of membranes, and premature delivery **(Oakeshott *et al.,* 2010).** *C. trachomatis* is also a major threat to reproductive health in women and has also been linked to infertility and ectopic pregnancy **(Hafner *et al.,* 2015)**.

Earlier studies showed that bacterial chorioamnionitis influence protease release, which leads to the rupture of the membranes, arachidonic acid cascade activation and uterine contractions. The result of these effects can be premature rupture of the membranes, preterm delivery or miscarriage **(Thomas *et al.,* 1990; Rastogi *et al.,* 2000).** Many authors tried to establish the relationship between *C. trachomatis* infection and pregnancy outcomes, and their results seem to be more reliable thanks to the development

of the diagnostic methods, especially molecular ones. Various studies have established a relation between *C. trachomatis* infection and spontaneous abortions in women **(Rastogi *et al.,* 2000; Trojniel *et al.,* 2009; Jahromi *et al.,* 2010; Baud *et al.,* 2011; Ahmadi et al., 2016)**. Early *in vitro* studies proved that the amniotic epithelium is susceptible to *C. trachomatis* infection and damage **(Neeper *et al.,* 1990).** It was further showed that foetal infection by *C. trachomatis* is also possible and can cause miscarriage **(Thomas *et al.,* 1990).**

*C. trachomatis* antibodies were also found in miscarriage cases **(Witkin *et al.,* 1992 Baud *et al.,* 2007)**. Thus, past or chronic *Chlamydia* infections might increase the risk for miscarriage, even in the absence of a detectable current infection, potentially by triggering chronic inflammation. Current or past infection with *Chlamydia* species may cause adverse pregnancy outcomes via two potential pathogenic mechanisms that affect the integrity of the placenta. Inflammatory activation of endometrial immunocytes by infections can cause an excessive maternal immune response towards the trophoblastic invasion and induces an early pregnancy failure in some recurrent aborters. The immunological hypothesis postulates that the anti-*Chlamydia* immune response results in endometrial, placental and fetal damage. The cellular hypothesis suggests that cytokine release by persistently infected cells may directly cause pregnancy termination **(Baud *et al.,* 2008).**

Asymptomatically infected women may be at risk of serious reproductive squeal; therefore, screening for genital chlamydial infection is a high public health priority **(Toye *et al.,* 1998).** The increasing prevalence of chlamydial disease has generated much interest in development of sensitive, specific, and rapid techniques for diagnosis of

chlamydial infections **(Kessler *et al.,* 1994).** Diagnosis of *C. trachomatis* infection is frequently based on bacterial isolation in tissue culture media. This method requires careful specimen collection and stringent transport condition and requires at least 48 to 72 hour**.** However, culturing *C. trachomatis* is technically difficult, given its strict intracellular life cycle.

Several laboratory methods are used for the diagnosis of *C. trachomatis*, include cytological tests for the detection of intra-cytoplasmic inclusions, cell culture, immunoassays enzyme analysis, direct immunofluorescence, DNA hybridization tecniques and DNA amplification PCR **(Cristina *et al.,* 2002).** Nucleic acid amplification techniques, such as PCR method have been used to detect *C. trachomatis* infection. PCR has proved to be more sensitive and specific than the conventional microbiological assays **(Puolakkainen *et al.,* 1998).** PCR involve exponential amplification of well-defined DNA targets, resulting in enhanced sensitivity of detection compared with the sensitivities of other nonculture methods **(Toye *et al.,* 1998).** In addition, PCR appears to be an optimal tool for enhancement of the sensitivity of detection **(Kessler *et al.,* 1994).** The previous studies shows that PCR has a sensitivity of 97% to 100% and a specificity of 98% for detecting *C. trachomatis*, while culture has a sensitivity and specificity of 85% and 100%, respectively **(Wilcox *et al.,* 2000).**

The burden of *C. trachomatis* organisms in the genital tract can vary from 10 to over a million organisms per milliliter of genital tract secretions **(Michel *et al.,* 2007).** This is likely to influence the performance of different nucleic acid amplification tests, which do not routinely distinguish between people with high and low chlamydial loads **(Michel *et al.,* 2007).** Hence, a technological innovation that came from PCR, named

real-time PCR, has become more common for clinical diagnostics and in research labs, because of its ability to generate quantitative results. Real-time PCR quantifies DNA and RNA precisely and with greater reproducibility, because it determines values during the exponential phase of the reaction. Its sensitivity makes it possible to use a very small sample, with extremely small residues of blood or tissue even parts of a single cell **(Xia *et al.,* 2007).** The time needed to produce a result is approximately two hours; the other techniques need more time for processing the material.

Thus in the present study, we compared the rate of detection of *C. trachomatis* infection with gene specific PCR and also quantify the chlamydial load in the endometrial curettage tissue samples by real time PCR from patients found infected with *C. trachomatis.*

## 3.2. Results

### 3.2.1. Clinical characteristics of aborters

150 spontaneous aborters (SA) constituted the study group (group I). In group I, 49 women experienced one abortion were included as sporadic spontaneous aborters (SSA) while 101 with two or more abortions constituted the recurrent spontaneous aborters (RSA). The mean age of SA was 24$\pm$ 4.0 years and the average gestational age was 8 weeks (first trimester). The average gravidity was 2; however the mean parity was 2 in these women. The clinical characteristics of aborters in group I - II are summarized in **Table 3.1.**

**Table 3.1:** Clinical characteristics of aborters (Groups I–II)

| **Clinical Characteristics** | **Group I (Spontaneous aborters) N= 150 (%)** | **Group II (Induced aborters) N= 150 (%)** | **'*p*'-value** |
|---|---|---|---|
| **Age (years)** | | | |
| **<20** | 14 (9.3) | 13 (8.6) | 0.84 |
| **20-25** | 55 (36.7) | 55 (36.6) | 1.00 |
| **26-30** | 32 (21.3) | 29 (19.3) | 0.89 |
| **31-35** | 27 (18) | 32 (21.3) | 0.57 |
| **35-40** | 22 (14.7) | 21 (14.0) | 0.87 |
| | Mean age: 25 ± 4.0 | | |
| **Gravidity** | | | |
| **01** | 49 (32.7) | 40 (26.6) | 0.46 |
| **02** | 46 (30.7) | 52 (34.6) | 0.72 |
| **>2** | 55 (36.6) | 58 (38.6) | 0.82 |
| | Average Gravidity: 2<br>Average gestational age: 8 weeks | | |
| **Parity** | | | |
| **0-1** | 97 (64.7) | 44 (30.6) | 0.006 |
| **2->2** | 53 (35.3) | 106 (69.3) | 0.001 |
| **No. of Abortions** | | | |
| 0-1 | 49 (32.7) | - | < 0.0001 |
| 2- >2 | 101 (70.6) | - | < 0.0001 |

### 3.2.2. Detection of TORCH-associated microorganisms in sera of aborters by ELISA

Out of 150 women in group I, a total of 15 patients were found infected with TORCH-associated micro-organisms **(Table 3.2- 3.4)** and hence excluded from the study. Among the controls (group II), 8 out of 150 women were found positive with various TORCH infections, so they were also excluded.

**Results in units**

$$\frac{\text{Patient (mean) absorbance value x 10}}{\text{Cut-off}} = \text{[NovaTec Units]} = \text{NTU}$$

**Table 3.2: Interpretation of results of TORCH-associated micro-organisms by ELISA**

| | | |
|---|---|---|
| Cut-off | 10 NTU | - |
| Positive | > 11 NTU | Antibodies against the pathogen are present. |
| Equivocal | 9 – 11 NTU | Antibodies against the pathogen could not be detected clearly. (Recommended to repeat the test with a fresh sample in 2 to 4 weeks. If the result is equivocal again the sample is judged as negative). |
| Negative | < 9 NTU | The sample contains no antibodies against the pathogen. A previous contact with the antigen is unlikely. |

**Table 3.3:** Diagnostic sensitivities and specificities of ELISA kits

| Microorganism | Sensitivity | Specificity |
|---|---|---|
| *Toxoplasma gondii* | 95.8 % | > 98 % |
| Rubella Virus | 100 % | 100 % |
| Cytomegalovirus | 91 % | > 98 % |
| Herpes simplex virus | > 95 % | > 95 % |

**Table 3.4:** TORCH infection positivity in sera of group I / group II patients

| **TORCH-associated microorganisms** | **Group I (Study group)**<br>**N = 150** | **Group II (Control group)**<br>**N = 150** |
|---|---|---|
| Toxoplasma | 15 | 04 |
| Tox + Rub | 11 | 01 |
| Tox + Rub + CMV | 09 | 02 |
| Tox + Rub + HSV | 05 | 01 |
| **Total** | **15** | **08** |

Abbreviations: Tox - Toxoplasma, Rub - Rubella, CMV- Cytomegalovirus, HSV- Herpes simplex virus.

### 3.2.3. Molecular detection of *Chlamydia trachomatis* by endogenous plasmid/ MOMP and 16S rRNA gene-specific PCR in Groups I- II

**Among 150 SA (group I), 27 were found to be positive for *C. trachomatis* infection by gene-specific PCR assay for MOMP/ plasmid/ 16S rRNA genes. Thus, the overall *C. trachomatis* positivity in women undergoing incomplete spontaneous abortion (group I) was 18%.** Of these, 19/150 (12.7%) women were found *C. trachomatis*- positive with 517 bp plasmid gene **(Fig. 3.1)**, 18/150 (12%) were positive with 200 bp plasmid gene **(Fig. 3.2)** while *C. trachomatis* MOMP DNA was detected in 15/150 (10%) women in group I **(Fig. 3.3)**. Furthermore, 9/150 (6%), were found to be positive for *C. trachomatis* 16S rRNA gene **(Fig. 3.4, Table 3.5). Among the controls (group II), 4/150 (2.6 %) patients were found positive with *C. trachomatis* infection and were hence excluded from the study (Table 3.6).** The percent *C. trachomatis* positivity was significant from group I in comparison to group II controls (Fisher exact test; 'p' value < 0.0001) **(Fig. 3.5 a).**

Furthermore, among the study group, 7/49 (14.3%) SSA were *C. trachomatis* positive and 15/101 (14.8%) RSA were infected with either MOMP/ plasmid/ 16S rRNA genes, which was not significant ('p' value > 0.05) **(Fig. 3.5 b)**.

**In group I,** 27 SA were *C. trachomatis*; among the latter, 5 were co-infected with TORCH and were hence excluded from the study. So, further immunomolecular studies were conducted in **22 *C. trachomatis*-positive SA**. In the remaining 123 *C. trachomatis*-negative SA, 10 were co-infected with TORCH, hence excluded from group I, and the remaining ***C. trachomatis*-negative SA served as internal controls. In group II**, 4 women were found to be TORCH-positive and excluded, so further studies were

performed in the remaining ***C. trachomatis*-negative controls.** No significant differences in *C. trachomatis* prevalence were observed when subjects were classified by history of recurrent abortions or gestational age.

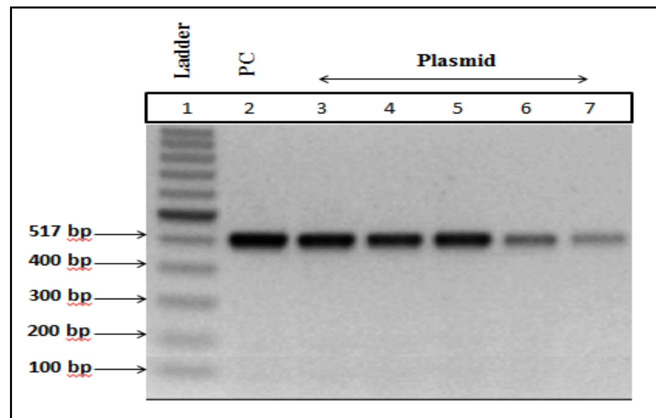



**Fig 3.1:** PCR for endogenous plasmid (517 bp) of *Chlamydia trachomatis* in the ECT of spontaneous aborters. Lane 1 shows 100 bp ladder, lane 2 shows positive control (PC), while lanes 3 - 7 show *Chlamydia trachomatis*-positive DNA samples in ECT.

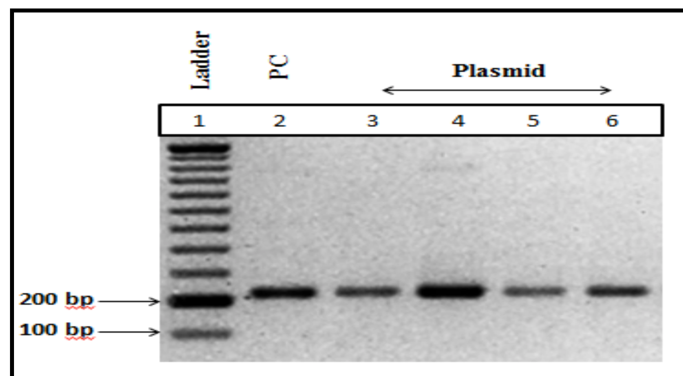



**Fig 3.2:** PCR for endogenous plasmid (200 bp) of *Chlamydia trachomatis* in the ECT of spontaneous aborters. Lane 1 shows 100 bp ladder, lane 2 shows positive control (PC), while lanes 3 - 6 show *Chlamydia trachomatis*-positive DNA samples in ECT.

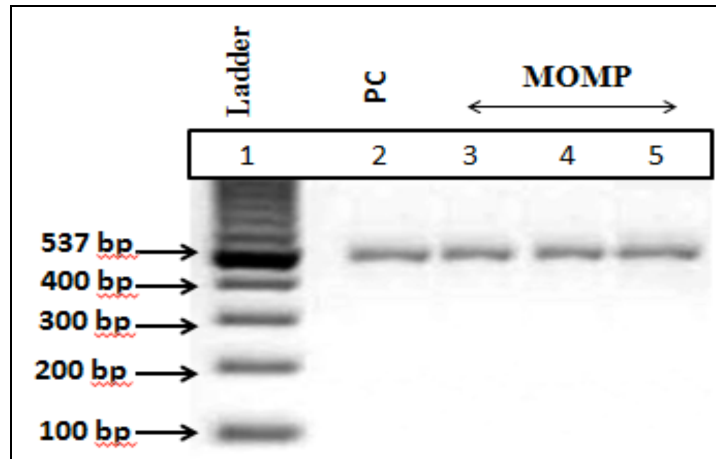



**Fig 3.3:** PCR for MOMP (537 bp) gene of *Chlamydia trachomatis* in the ECT of spontaneous aborters. Lane 1 shows 100 bp ladder, lane 2 shows positive control (PC) for *Chlamydia trachomatis* DNA while lanes 3- 5 show *Chlamydia trachomatis*-positive samples in ECT.

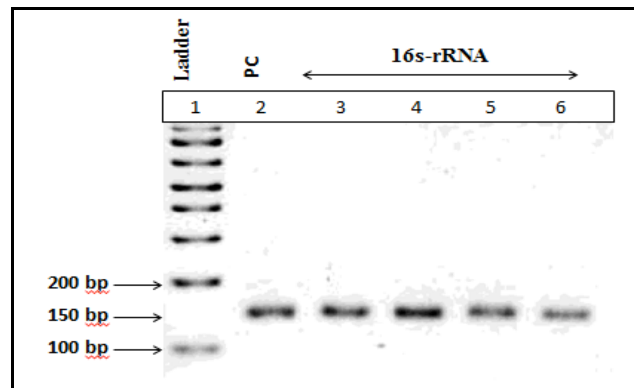
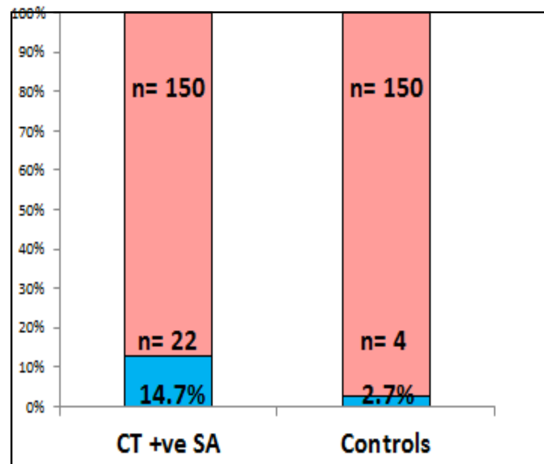



**Fig 3.4:** PCR for 16S rRNA (150 bp) gene of *Chlamydia trachomatis* in the ECT of spontaneous aborters. Lane 1 shows 100 bp ladder, lane 2 shows positive control (PC) for *Chlamydia trachomatis* DNA while lanes 3- 6 show *Chlamydia trachomatis*-positive samples in ECT.

**Table 3.5:** Detection of *Chlamydia trachomatis* by PCR assay in the Group I women

| **No. of Samples** | **Diagnostic methods in endometrial curettage tissues** | | | |
|---|---|---|---|---|
| | Plasmid PCR (517 bp) | Plasmid PCR (200 bp) | MOMP PCR (537 bp) | 16S rRNA (150 bp) |
| 01 | + | - | - | + |
| 03 | - | - | + | + |
| 05 | + | + | + | + |
| 06 | + | + | - | - |
| 07 | + | + | + | - |
| Total positives | 19 | 18 | 15 | 09 |

**(a)**




**(b)**

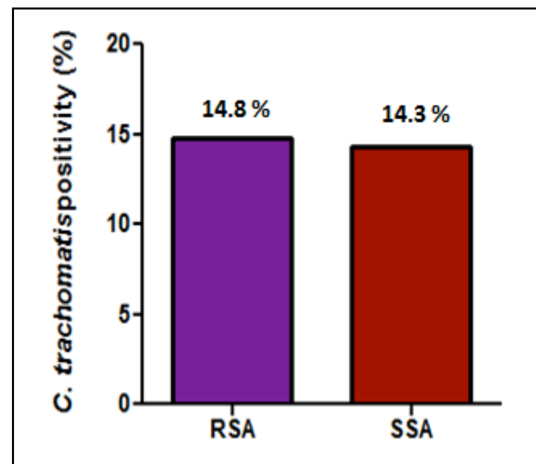



**Fig. 3.5: a.** *Chlamydia trachomatis* detection in ECT by PCR in SA (Group I) and in control group (Group II) (*'p'* = < 0.05) **b.** *Chlamydia trachomatis* positivity in RSA versus SSA sub-group in ECT samples by gene-specific PCR in Group I (*'p'* = non-significant).

**Table 3.6:** *Chlamydia trachomatis* positivity in Group I-II

| Patient group | No. of patients | *C. trachomatis* +ve | *'p'*-value |
|---|---|---|---|
| **Group I (Study group)** | n= 150 | n= 22 (14.7) | <0.05 (S) |
| SSA | | | |
| RSA | n= 49 | n= 07 (14.3) | |
| | n= 101 | n= 15 (14.8) | |
| **Group II (Controls)** | n= 150 | n= 04 (2.7%) | |

SSA: Sporadic Spontaneous Aborters; RSA: Recurrent Spontaneous Aborters

### 3.2.4. Identification of PCR products by DNA sequencing

PCR products of *C. trachomatis*-positive patients were purified and sequenced. The resulting DNA sequences were compared with sequences in the NCBI database using BLAST. DNA findings to *C. trachomatis* pathogen had percent similarity > 98%, the best score and the maximum length of alignment between the sequenced positive PCR product and the corresponding sequence in the database.

### 3.2.5. Quantitative detection of *Chlamydia trachomatis* by real time PCR

Further, real time PCR was performed by using the SYBR green based chemistry to quantitate the *C. trachomatis* copies in ECT of groups I- II. Standard curve was obtained by preparing the serial dilutions of *C. trachomatis* positive control as discussed in chapter 2. Cycle threshold (Ct) values of all the samples were calculated by q-PCR and the copy number of *C. trachomatis* in each sample was evaluated by using the standard curve, plotted by using the known concentrations of *C. trachomatis* positive control. As

q-PCR is capable of picking up even a single copy of *C. trachomatis,* a total 1- 8 copies were detected in *C. trachomatis*-positive patients **(Fig. 3.6).**

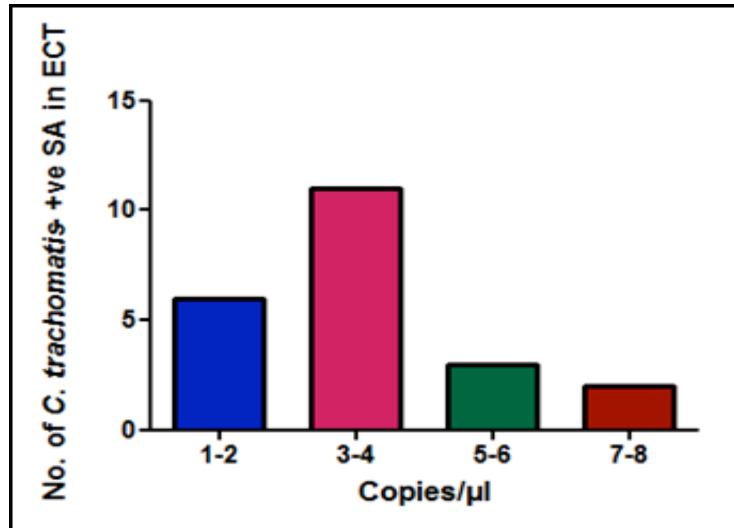



**Fig. 3.6:** Distribution of *Chlamydia trachomatis* DNA copy load in ECT samples of Group I women by plasmid gene specific Real time PCR.

### 3.3. Discussion

Bacterial infections can result in foetal loss **(McDuffie *et al.,* 1992)** and *C. trachomatis* is a major threat to reproductive health in women, and has been implicated in a substantial proportion of cases of subfertility and adverse pregnancy outcomes **(Rastogi *et al.,* 2000; Baud *et al.,* 2011).** The first concern that there might be a link between *Chlamydia* species and spontaneous abortion was derived from the observation that *C. psittaci* caused fetal wastage in bovine species and, thereafter, from case reports that farm women had spontaneous abortions after contact with an infected sheep or lamb **(Johnson *et al.,* 2002)**. Subsequently, a handful of initial studies attempted to establish a relationship between serological evidence of past *C. trachomatis* infection and

spontaneous abortion. These investigations were conducted among women who had experienced recurrent pregnancy loss **(Osser *et al.,* 1996)**.

*C. trachomatis* has been isolated or detected in cervical smear, urine and placental tissue **(Wilkowska-Trojniel *et al.,* 2009; Baud *et al.,* 2011).** Previous studies have shown the prevalence of chlamydial infection in India **(Malik *et al.,* 2009; Becker *et al.,* 2010).** The relationship between the presence of antichlamydial antibodies in serum and recurrent abortions was confirmed (**Witkin *et al.,* 1992)**. In women who experienced miscarriage, *C. trachomatis* infection was confirmed in 39.3% and in 33.2% respectively **(Osser *et al.,* 1996).** NAAT showed that the prevalence of genital *Chlamydia* infections amongst pregnant women was found to be 0.1% **(Vidwan *et al.,* 2012).** Also, when used to detect asymptomatic *Chlamydia* in all types of samples, NAATs have been reported to be more sensitive than non–NAATs **(Watson *et al.,* 2002).** In one study, the prevalence of *C. trachomatis* in patients with spontaneous pregnancy loss was 32% by using PCR **(Magon *et al.,* 2005).** In another study, 21.3% *C. trachomatis* positivity was found in women with abortion detected by PCR **(Zeighami *et al.,* 2008).** In one study, positive IgG serology for *C. trachomatis* was associated with recurrent early miscarriages but not with late miscarriages (12–24 weeks) or stillbirths **(Baud *et al.,* 2007).** A higher prevalence of *C. trachomatis* antibodies in spontaneous or recurrent miscarriages have been reported by several other groups **(Vigil *et al.,* 2002; Baud *et al.,* 2007)**. Our study showed the presence of genitourinary tract *C. trachomatis* infection in the ECT of 14.7% patients and 14.3% with one spontaneous abortion (SSA) and 14.8% of women with two or more abortions (RSA) showing that persistant *C. trachomatis* infection can cause fetal tissue damage leading to spontaneous abortion in women. Our findings are consistent

with previous reports stating that early pregnancy loss could be induced by persistent, asymptomatic *C. trachomatis* infection spreading to the fetal tissue **(Gravett *et al.*, 1986).**

Even with molecular approaches, detecting *C. trachomatis* can be difficult because of PCR inhibitors or low number of copies often present in the lesions **(Penta *et al.*, 2003; Wilkowska-Trojniel *et al.*, 2009)**. Genital infection with *C. trachomatis* leads to the presence of copies of target DNA in ECT and is detectable by standardized PCR and can be further quantified by using quantitative real time PCR. In the current study, we have quantified the chlamydial load in the ECT specimens by using MOMP gene real time PCR and it has been able to detect even a single copy of *C. trachomatis* in few samples and a total of 1-8 copies of *C. trachomatis* were quantified.

Based on our results, the study showed that *C. trachomatis* infection has to be taken into consideration as far as pregnancy failures/ spontaneous abortions are concerned. Molecular diagnostic methods, such as PCR and real time PCR based on specific genes detection seem to be important in diagnosing *C. trachomatis* infections in such patients.

# CHAPTER 4

# EXPRESSION OF CYCLOOXYGENASES, PROSTAGLANDIN RECEPTORS AND HORMONE LEVELS IN *CHLAMYDIA TRACHOMATIS*-INFECTED SPONTANEOUS ABORTERS

## 4.1. Introduction

Spontaneous abortion is a frequent outcome in pregnancy and is one of the most difficult areas in reproductive medicine, since the etiology is often unknown. Currently, the role of infectious agents in pregnancy loss is not clear and is under active investigation. Although several studies described about an association between *Chlamydia trachomatis* infection and spontaneous abortion**,** the underlying immunomolecular pathway leading to spontaneous abortion in *C. trachomatis* infected women is not yet clear.

In vitro studies revealed the expression of COX-1 and COX-2 in uterine tissues under different physiological states such as recognition of pregnancy and parturition. In response to bacterial infections, COX-2 induces and regulates the prostaglandin (PG) formation. PGs could be involved in the maintenance of uterine quiescence for the majority of gestation **(Challis *et al.,* 2002).** Since PG stimulate myometrium and are capable abortifacients **(Hertelendy & Zakar, 2001)**, they are likely to be important mediators of LPS-induced pregnancy loss. LPS induced abortion has also been found associated with PGs **(Skarnes & Harper, 1972).** Data published till date has focussed on the expression of COX-2 during recurrent pregnancy loss, preterm birth, foetal death, etc. in uninfected women **(Yu *et al.,* 2010).**

Furthermore, abnormal systemic endocrine disorders have been suggested as being associated with recurrent miscarriages, including luteal phase defects and hypersecretion of luteinizing hormone **(Regan *et al.*, 1990).** The term ‘luteal phase defect’ refers to the functional inadequacy of the corpus luteum to produce appropriate amounts of progesterone. This results in inadequate endometrial maturation and probable

functional defects in the early maintenance of the implanting embryo **(Serle *et al.*, 1994).** The significance of progesterone is illustrated in women without functional ovaries undergoing oocyte donation who, after receiving oestrogen and progesterone treatment, achieve successful pregnancies **(Crosignani, 1988).** Endocrine factors like progesterone and estrogen have also been implicated in the etiology of spontaneous abortion, with poorly understood roles. In pregnancy, progesterone is in dynamic balance with estrogen in the control of uterine activity. A fetal endocrine cascade involving an increase in estrogen and decrease in progesterone in maternal plasma trigger the onset of labour **(Challis *et al.,* 2000).** This endocrine cascade ultimately leads to both the activation and stimulation of the myometrium through the increased production of stimulatory PGs. Thus, low progesterone values are associated with miscarriage and ectopic pregnancy, both considered as non-viable pregnancies.

Although the significant association between *C. trachomatis* and spontaneous abortion is clearly evident, yet the underlying mechanism responsible for spontaneous abortion still remains to be established and probably, gene expression, particularly the role of COX and its signalling pathway in *C. trachomatis*-infected aborters might be the key mechanism. Till date, there are no studies on the expression pattern of COX-2 and contractile/ relaxatory PG receptors during spontaneous abortion in *C. trachomatis*-positive women, hence the present study aims to quantify the mRNA expression of PG contractile/ relaxatory receptor genes and seeks to find the effect of gestational age (GA) on the expression of PG receptors in *C. trachomatis*-positive SA. Also serum hormonal levels were estimated in these patients to establish a correlation between the PG receptors expression and hormones in *C. trachomatis*-positive SA.

## 4.2. Results

### 4.2.1. Qualitative analysis of mRNA expression for cyclooxygenases (COX-1/ COX-2) genes by reverse-transcription PCR

Expression of COX-1 and COX-2 genes in the ECT was checked by performing RT- PCR in relation to the housekeeping gene, *viz.:* beta actin in *C. trachomatis*-positive patients in comparison to the controls. In all *C. trachomatis*-positive endometrial tissue specimens, clear signals were detected for the cDNA amplification products of the COX-1 and COX-2 genes **(Fig. 4.1 a- b).** The COX-2 gene expression was found significantly higher in *C. trachomatis*-positive SA in comparison to the *C. trachomatis*-negative control group, however the expression of COX-1 was not significant in the study group in comparison to the controls, ($p < 0.0001$; arbitrary units; **Fig. 4.2 a-b**).

**(a)** **(b)**

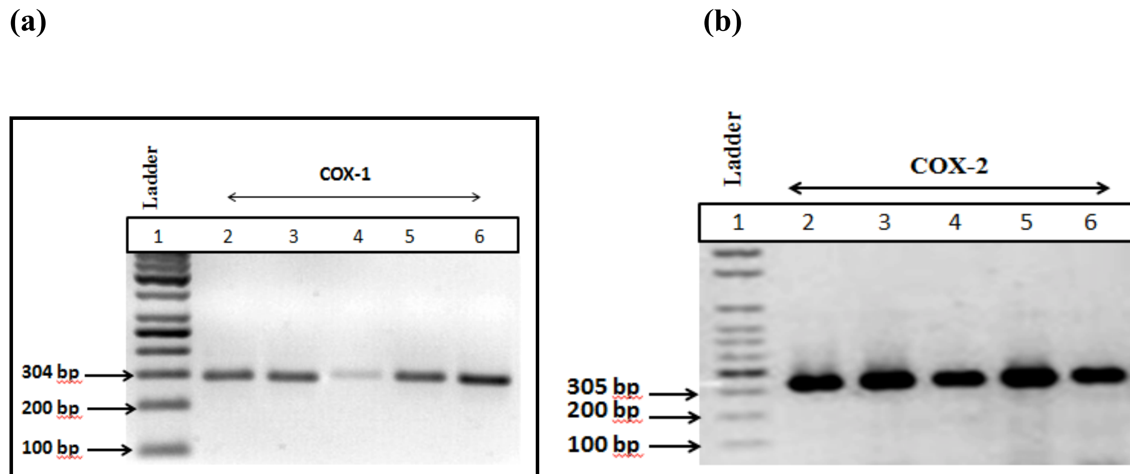



**Fig. 4.1:** Amplification products for COX in ECT of *Chlamydia trachomatis*-positive spontaneous aborters; **(a)** is showing the amplification products of COX-1; **(b)** shows the amplification products of COX-2; Lane - 1 shows 100 bp ladder, lanes 2 - 6 show COX-1/ COX-2 expression in the ECT of *Chlamydia trachomatis*-positive samples.

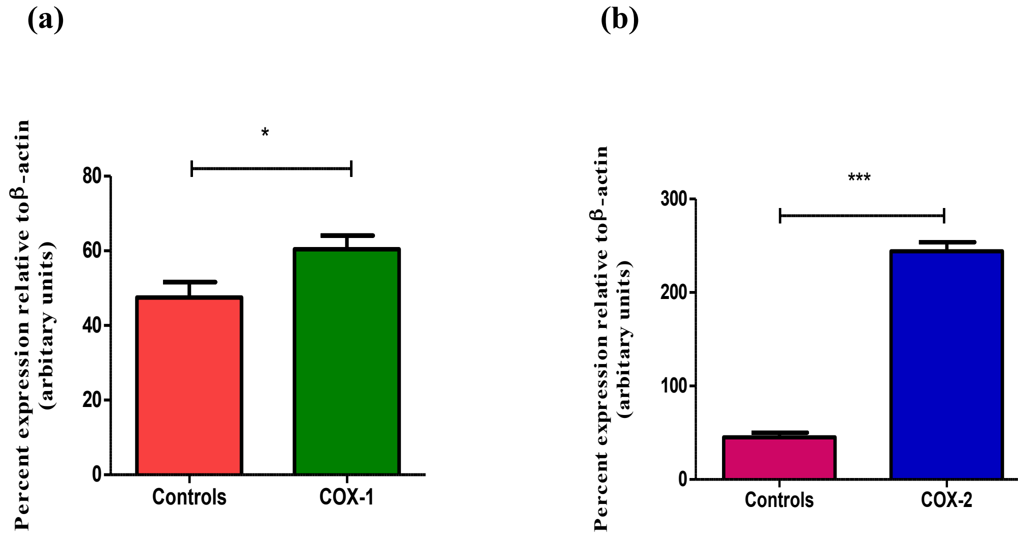



**Fig. 4.2: (a-b)** Expression of COX-1 and COX-2 genes in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group by RT-PCR (Non-parametric Mann-Whitney test '*p*' < 0.05 for COX-1 and '*p*' < 0.0001 for COX-2).

### 4.2.2. Qualitative analysis of mRNA expression for gene encoding receptors (EP-1, EP-2, EP-3, EP-4, FP, IP) of PGE2, PGF2α and PGI2 by reverse transcription PCR

Qualitative expression of EP-1, EP-2, EP-3, EP-4, FP and IP receptors in the ECT was checked by performing RT-PCR in relation to beta actin gene in *C. trachomatis*-positive patients in study group in comparison to the controls by using RT-PCR assay. In all *C. trachomatis*-positive endometrial tissue specimens, clear signals were detected for the cDNA amplification products of the EP-1, EP-2, EP-3, EP-4, FP and IP receptor genes **(Fig. 4.3).** The expression of all the PG receptors was found significantly higher in *C. trachomatis*-positive SA in comparison to the control group ((Non-parametric Mann-Whitney test '*p*' < 0.0001; arbitrary units; **Fig. 4.4**).

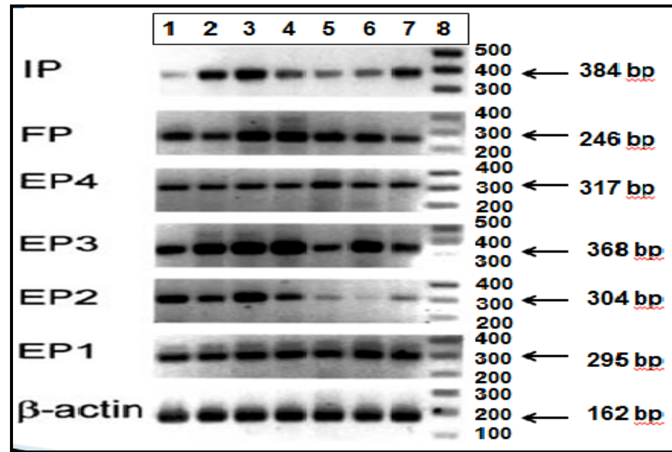



**Fig. 4.3:** Amplification products for EP-1, EP-2, EP-3, EP-4, FP and IP receptors in ECT of *Chlamydia trachomatis*-positive spontaneous aborters. Lanes 1 - 7 show expression of PG receptors in the ECT while Lane- 8 shows 100 bp ladder.

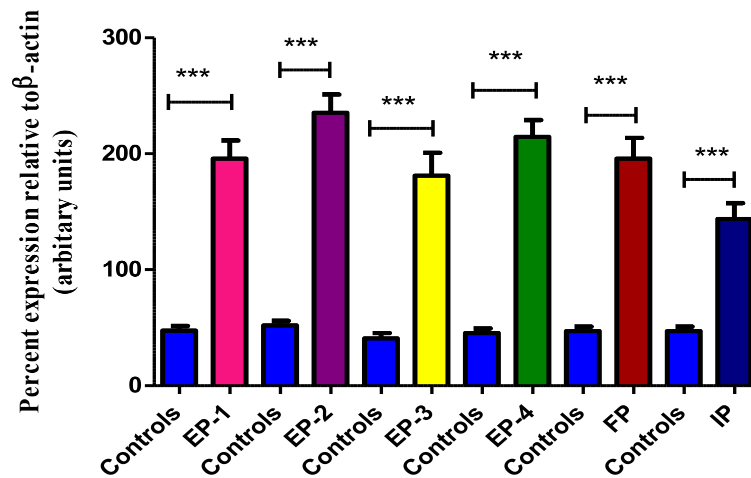



**Fig. 4.4:** Increased expression of EP-1, EP-2, EP-3, EP-4, FP and IP receptor genes in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group (Non-parametric Mann-Whitney test; '*p*'< 0.0001).

### 4.2.3. Quantitative analysis of mRNA expression for cyclooxygenases (COX-1/ COX-2) genes by quantitative real time PCR

Analysis of mRNA expression for COX-1 and COX-2 genes within the ECT by quantitative real time PCR was compared among patient group as a ratio to the expression of the constitutively expressed GAPDH. The expression of COX-1gene was neither significant in the *C. trachomatis*-positive SA nor in *C. trachomatis*-negative SA in comparison to the controls and its expression did not change with the gestational age **(Fig. 4.5 a-b).**

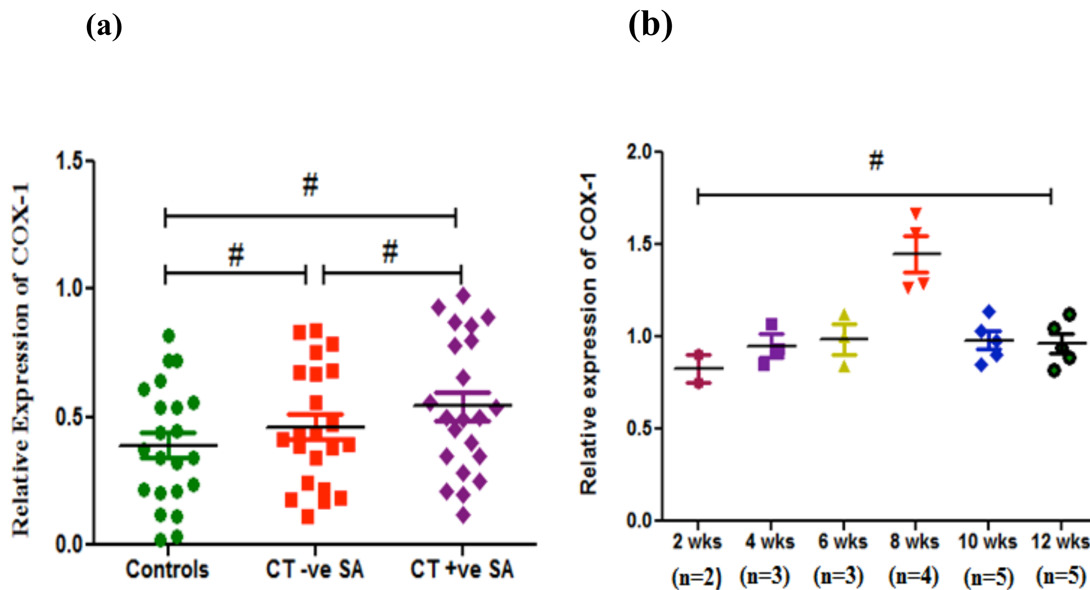



**Fig. 4.5: (a)** Expression of COX-1 gene was low in *C. trachomatis* positive spontaneous aborters (Group I) and was non-significant in comparison to controls (Non-parametric Mann-Whitney test; '*p*'> 0.05) **(b)** Cox-1 mRNA expression in ECT did not change with gestational age (One-way ANOVA, non-parametric Kruskal-Wallis test '*p*' > 0.05). ('*p*' value # = non-significant).

COX-2 mRNA expression demonstrated a significant increase in *C. trachomatis*-positive SA and in also uninfected SA (Group I) in comparison to Group II control women, though the expression was significantly high in *C. trachomatis*-positive SA versus uninfected SA (‘*p*’ < 0.0001; **Fig. 4.6 a**). Upon analysis of Ct values, the COX-2 gene was found 2.3-fold upregulated in *C. trachomatis*-infected women undergoing spontaneous abortion. ANOVA showed a significant effect of GA on COX-2 expression in the first trimester SAs (‘*p*’ < 0.0001; **Fig. 4.6 b**). The expression of COX-2 was also compared between the RSA and SSA groups and it was found that COX-2 was significantly higher in the RSA in comparison to the SSA (Mann-Whitney test, ‘*p*’ < 0.002; **Fig. 4.6 c**).

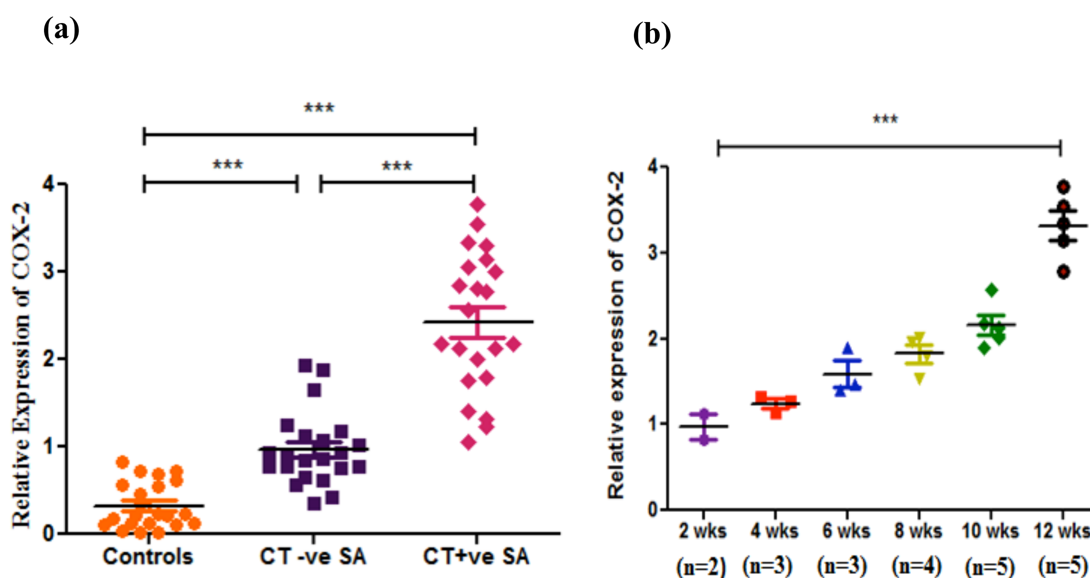



**Fig. 4.6: (a)** Expression of COX-2 gene was significantly high in *C. trachomatis*-positive spontaneous aborters (Group I) (Non-parametric Mann-Whitney test; ***‘*p*’ < 0.0001); **(b)** Expression of COX-2 in advanced gestational age (One-way ANOVA, non-parametric Kruskal-Wallis test ***‘*p*’ < 0.0001).

(c)

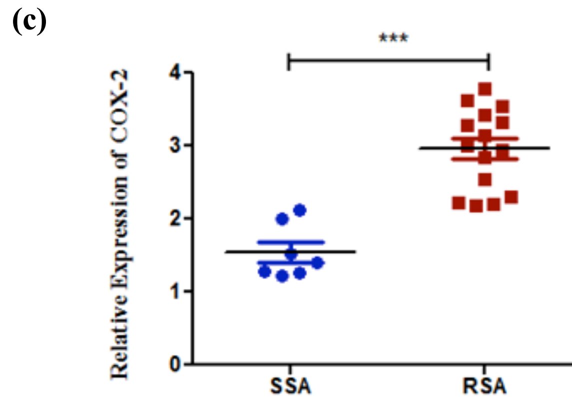



**Fig. 4.6: (c)** Expression of COX-2 gene was significantly high in RSA *versus* SSA (Non parametric Mann-Whitney test; ***‘*p*’ < 0.0001).

2.6.2. **Quantitative analysis of mRNA expression for PGE2, PGF2α and PGI2 gene receptors (EP-1, EP-2, EP-3, EP-4, FP, IP) by real time PCR**

In order to investigate whether chlamydial infection affects the functionality of the PG contractile/ relaxatory receptors, the expression of PGE2 (EP-1, EP-2, EP-3, EP-4), PGF2α (FP) and PGI2 (IP) receptor genes was studied in the ECT at transcript level by taqman q-PCR and the expression was compared among patient group as a ratio to the expression of the constitutively expressed housekeeping β-actin and GAPDH gene in *C. trachomatis*-positive SA. Analysis of Ct values showed that the mRNA expression of PGE2 contractile receptors EP-1/ EP-3 and relaxant receptors EP-2/ EP-4 was significantly increased in comparison to controls and uninfected SAs. The expression of all PGE2 receptors was also significantly high in *C. trachomatis*-negative SA in comparison to the controls (non-parametric Mann-Whitney test; ‘*p*’ < 0.0001) **(Fig. 4.7 a-d).**

Analysis of the gene expression of PGF2α contractile receptor FP showed that the gene expression was significantly higher in *C. trachomatis*-positive SA *versus* controls and *C. trachomatis*-negative SA (non-parametric Mann-Whitney test; ‘*p*’ < 0.0001) **(Fig. 4.7 e).** The PGI2 relaxant receptor gene, IP was also found increased significantly in the infected patients in comparison to controls (non-parametric Mann-Whitney test; ‘*p*’ < 0.0001) **(Fig. 4.7 f).**

A significant effect of GA was observed on the mRNA expression of various PGE-2, *viz.:* EP-1, EP-2, EP-3, EP-4, PGF2α (FP) and PGI2 (IP) receptor genes in the infected women. The expression of PG receptors was found to be increased significantly with the increasing GA in *C. trachomatis* positive spontaneous aborters (one-way ANOVA non-parametric Kruskal-wallis test; ‘*p’* < 0.002) **(Fig. 4.8 a-f).**

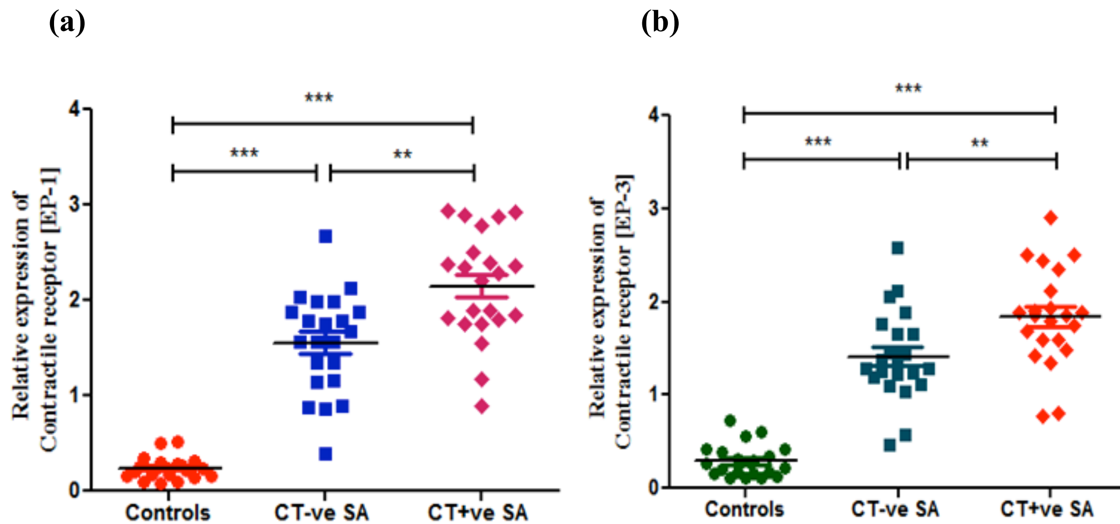



**Fig. 4.7: (a-b)** Expression of PGE2 Contractile receptor genes (EP-1, EP-3) in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group and *Chlamydia trachomatis*-negative spontaneous aborters by q-PCR (Non-parametric Mann-Whitney test; ***‘*p*’ < 0.0001). (‘*p*’ value *** = < 0.0001; ** = < 0.001).

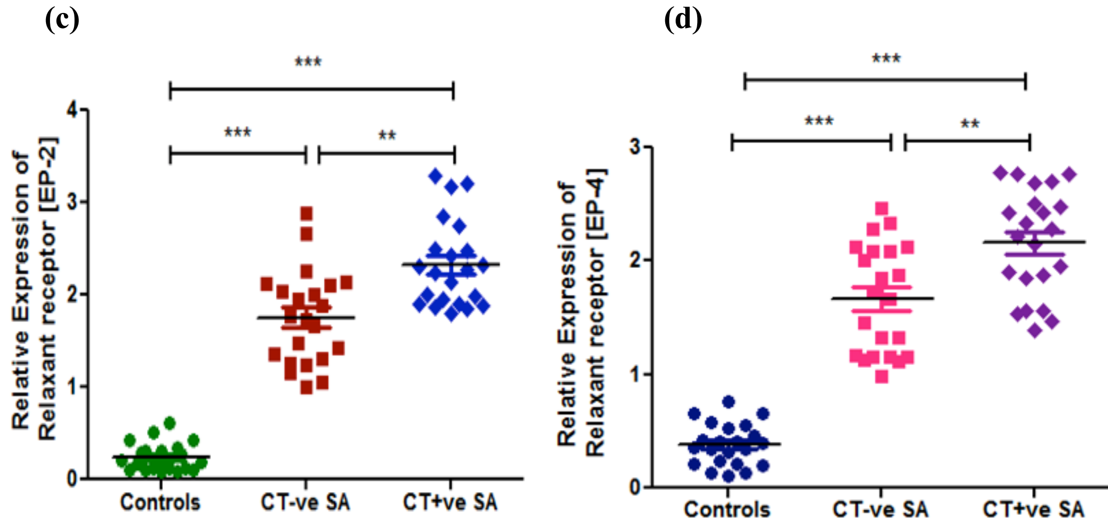



**Fig. 4.7: (c-d)** Expression of PGE2 Relaxant receptor genes (EP-2, EP-4) in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) by q-PCR (Non-parametric Mann-Whitney test; ***'*p*' < 0.0001). ('p' value *** = < 0.0001; ** = < 0.001).

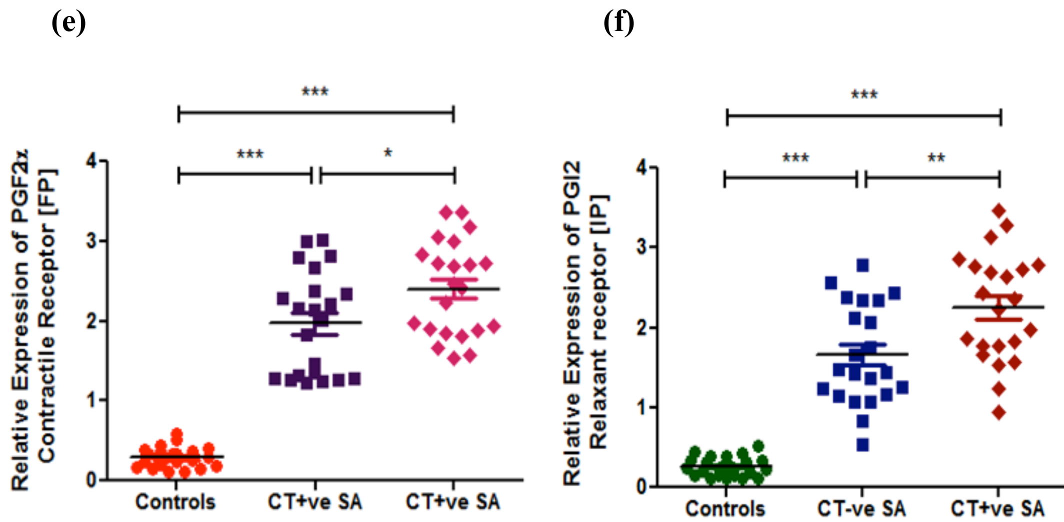



**Fig. 4.7: (e-f)** Expression of $PGF_{2\alpha}$ contractile receptor FP and $PGI_2$ Relaxant receptor IP by Real time PCR in *Chlamydia trachomatis*–positive spontaneous aborters (n = 22; Non-parametric Mann-Whitney test; ***'*p*'< 0.0001). ('*p*' value *** = < 0.0001; ** = < 0.001, * = < 0.001). **Abbreviations:** SA – spontaneous aborters; CT – *Chlamydia trachomatis.*

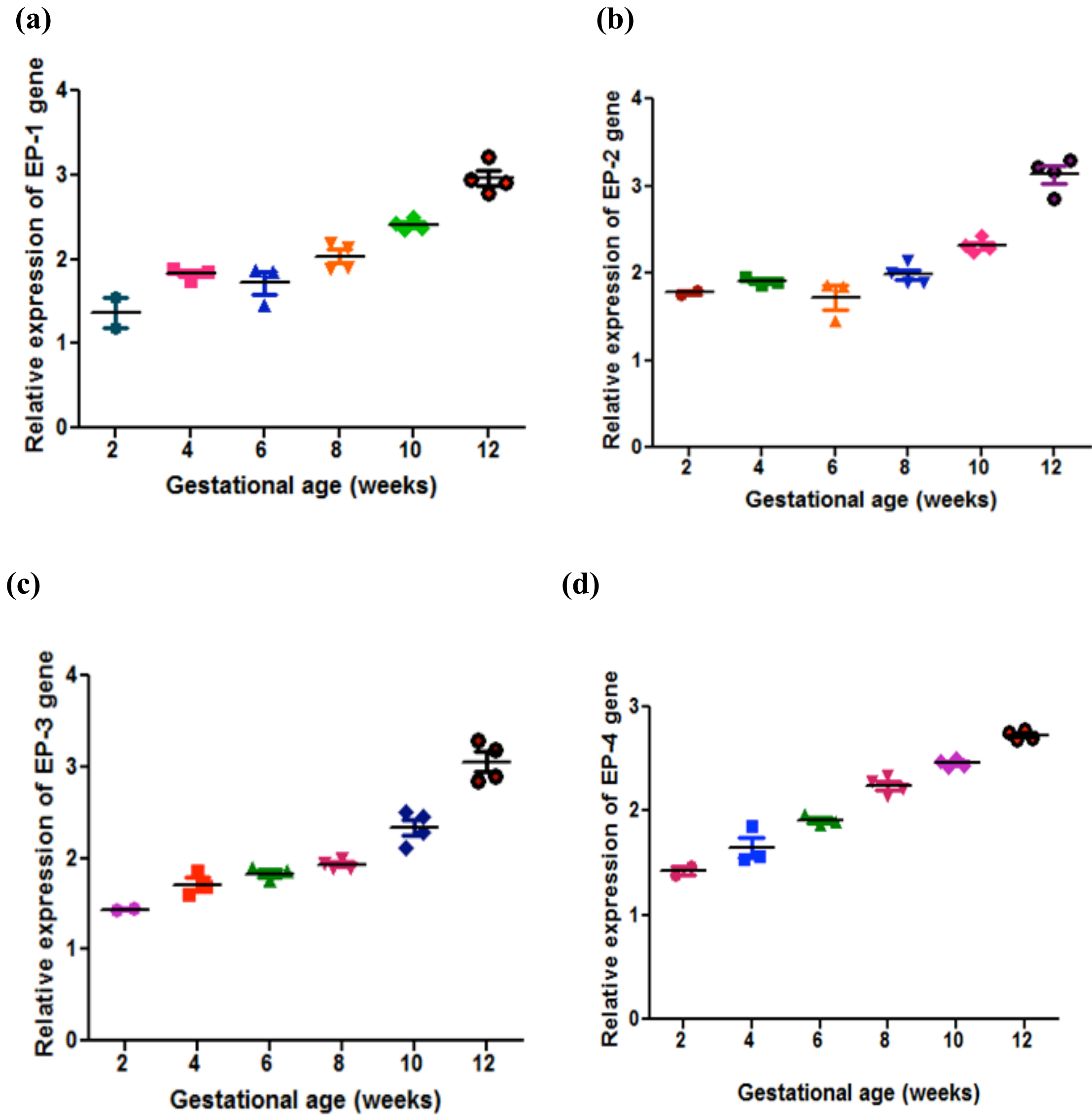



**Fig. 4.8:** **(a-d)** Expression of PGE2 receptors with advanced gestational age by Real time PCR in *Chlamydia trachomatis*–positive spontaneous aborters (n=22) (One way ANOVA, non-parametric Kruskal-Wallis test **'*p*' < 0.002).

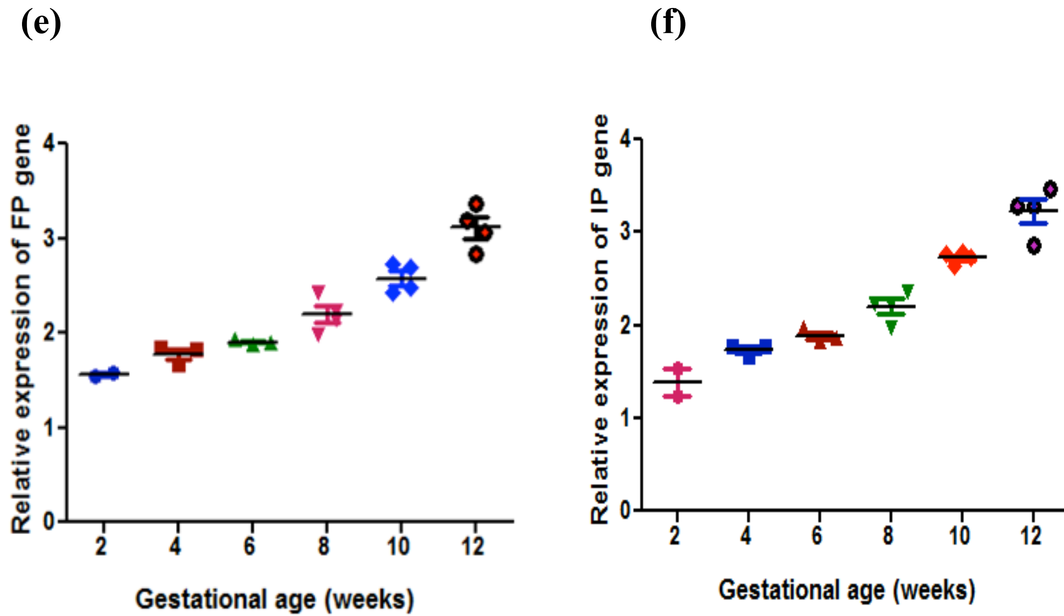



**Fig. 4.8:** (**e-f**) Expression of FP and IP receptor genes in advanced gestational age (One way ANOVA, non-parametric Kruskal-Wallis test **'*p*' < 0.002).

### 4.2.5. Correlation of PG receptors expression with gestational age

The PG receptor genes (EP-1, EP-2, EP-3, EP-4, FP and IP) were further correlated with GA. The 'r' value was found to be significant for all the receptors. The contractile EP-1 and EP-3 receptors were positively correlated (r = 0.903; '*p*' = 0.0028 and r = 0.96; '*p*' = 0.0021, respectively) **(Figs. 4.9 a, 4.9 c)** while the relaxant EP-2 and EP-4 receptors were found negatively correlated with GA (r = - 0.94; '*p*' = 0.017 and r = - 0.98; '*p*' = 0.0008, respectively) **(Figs. 4.9 b, 4.9 d)** in infected RSA. Similar correlation was also made between the FP receptor and GA in *C. trachomatis*-infected spontaneous aborters (Group I). The FP gene was found significantly positively correlated with gestational age (r = 1.00; '*p*' = 0.002) **(Fig. 4.9 e),** however, the IP receptor gene showed no correlation.

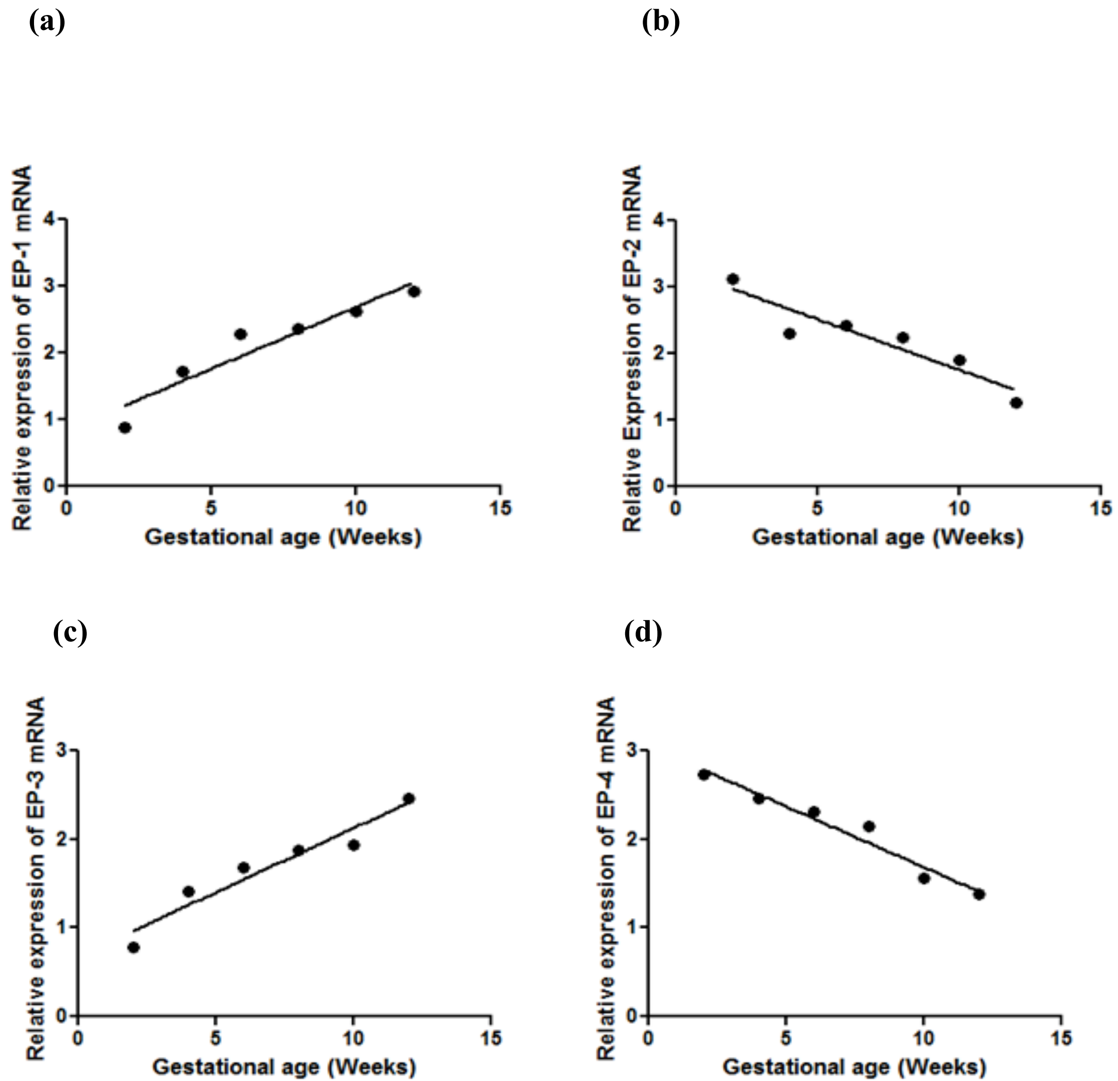



**Fig. 4.9:** Correlations made with Spearman rank correlation test between EP receptors and gestational age levels in spontaneous aborters with *C. trachomatis* infection **(a)** EP-1 and gestational age levels; r = 0.903, ** '*p*' = 0.0028; **(b)** EP-2 and gestational age levels; r = - 0.94, * '*p*' = 0.017; **(c)** EP-3 and gestational age levels; r = 0.96, ** '*p*' = 0.0021; **(d)** EP-4 and gestational age levels, r = - 0.98, *** '*p*' = 0.000.

**(e)**

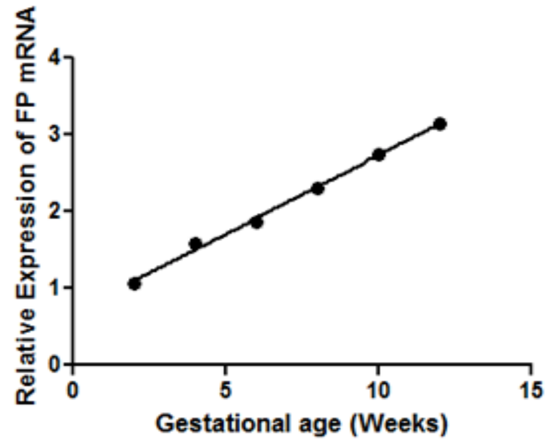



**Fig. 4.9: (e)** Correlations between FP and gestational age levels in spontaneous aborters with *C. trachomatis* infection. r = 1.00, ** '*p*' = 0.002.

### 4.2.6. Estimation of serum progesterone and estrogen in aborters

Mean serum estrogen concentration was significantly high (458.26 pg/ ml) in the *C. trachomatis*-positive SA, as compared to both uninfected SA (218.58 pg/ ml, Mann-Whitney test, '*p*' < 0.0001) and the control group (65.56 pg/ ml; Mann-Whitney test, '*p*' < 0.0001) **(Fig. 4.10 a).** Mean serum progesterone level was estimated in controls, *C. trachomatis*-positive as well as in uninfected SA and it was found that the progesterone concentration was significantly low (20.2 ng/ ml) in the *C. trachomatis*-positive SA, as compared to both uninfected SA (40 ng/ ml, Mann-Whitney test, '*p*' < 0.0001) and the control group (145.48 ng/ ml; Mann-Whitney test, '*p*' < 0.0001) **(Fig. 4.10 b).**

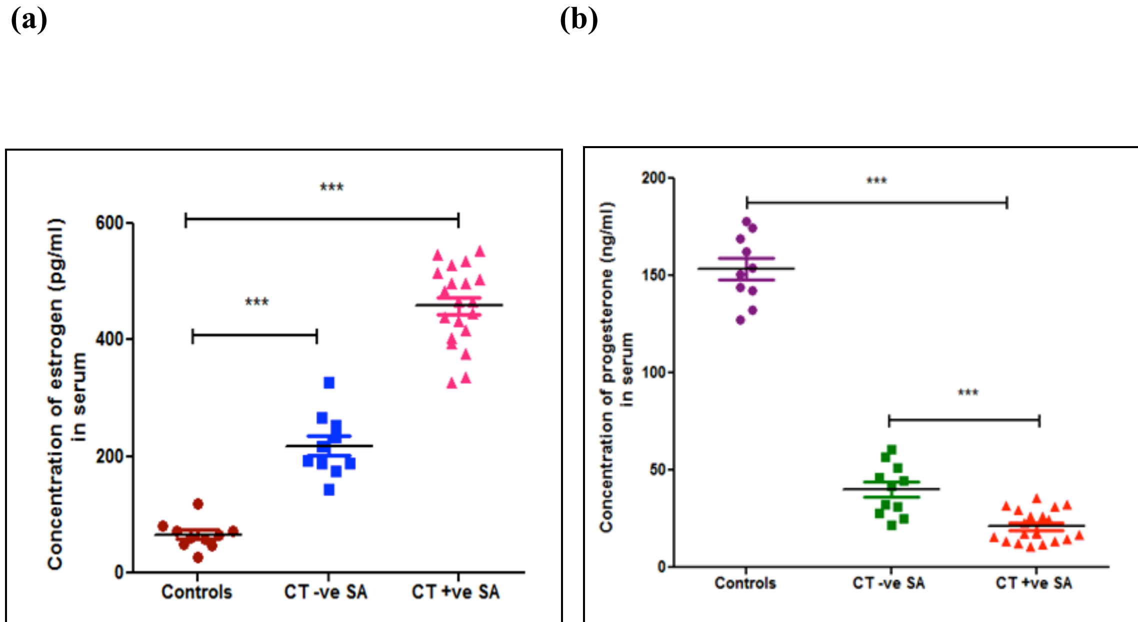



**Fig. 4.10: (a)** Estimation of Estrogen in the serum of aborted patients (Mann-Whitney test; ***$p < 0.0001$) **(b)** Estimation of Progesterone in the serum of aborted patients (Mann-Whitney test; ***$p < 0.0001$). **Abbreviations:** SA – spontaneous aborters; CT – *Chlamydia trachomatis.*

### 4.2.6. Discussion

Globally, *C. trachomatis* infection ranks among the most common sexually transmitted infections, and cause notable reproductive morbidity in women ranging from clinically inapparent disease to cervicitis, urethritis, endometritis, salpingitis/ infertility, pelvic inflammatory disease and ectopic pregnancy. The overall burden of morbidity and mortality due to chlamydial infections might be substantial therefore there is growing concern about the association of cervical *C. trachomatis* infection and poor obstetric outcome including still births, preterm deliveries, premature rupture of membranes, delivery of low birth weight infants and SA. In India, female genital *C. trachomatis*

infection is a major health problem because of increased prevalence **(Rastogi *et al.*, 2002).** Although the immunopathologic consequences of infection and the adverse effects that *C. trachomatis* has on the female genital tract are well established, the underlying mechanism of *Chlamydia*-induced spontaneous abortion in women warrants further research.

None of the studies till date has shown endometrial expression of COX-2 in infected women experiencing spontaneous abortions. Previous studies suggest that the expression of COX-2 regulates endometrial development and receptivity during implantation and early pregnancy and lower expression of COX-2 in chorionic villi is involved in unexplained recurrent spontaneous abortions **(Yu *et al.*, 2010).** The majority of COX-2 related studies have focussed on menstruation, preterm birth, foetal death, etc. however, there is virtually no report till date to the best of our knowledge on the role of COX-2 genes during abortion in infected women. It was also reported that LPS-induced decidual prostanoid production *via* increased COX-2 expression**.** In the case of bacterial infection, the production of pro-inflammatory cytokines further induces COX-2. The present study showed that the expression of COX-1 was neither found significant in *C. trachomatis*-positive SA nor in *C. trachomatis*-negative SA in comparison to the controls and COX-1 expression did not change with the SA. The expression of COX-2 increased significantly in *C. trachomatis*-positive SA and also in uninfected SA in comparison to control women, though the expression was significantly high in *C. trachomatis*-positive SA versus uninfected SA. A significantly higher expression of COX-2 in infected SA may be attributed to chlamydial infection. Our study suggested that chlamydial infection

leads to an upregulation of COX-2 in infected SA, which probably mediated an increased PG synthesis.

These PGs mainly regulate vascular physiologic functions such as angiogenesis and vascular tone in addition to cell proliferation, differentiation and immunomodulatory functions. The role of COX and PG receptors has been elucidated in pregnancy and it has been suggested that the upregulation of COX-2 mediates increased PG synthesis within the foetal membranes at term and much of the increase in COX-2 expression is probably a consequence of labour **(Slater *et al.,* 1999).** The PGE2 receptors are present in the human myometrium tissue during pregnancy and at term, levels of both systemic and local PGE2 increase dramatically **(Leonhardt *et al.*, 2003).** PGE2 is generated in abundance at the sites of infection and inflammation as a result of the rapid upregulation of COX-2 **(Koeberle & Werz, 2009).** PGF2α has also been considered as the primary candidate present during pregnancy, where it plays a crucial role in the myometrium during parturition by increasing the oxytocin-induced contractions. Furthermore, an increase in intrauterine PGF2α concentration in pregnant mice treated with LPS-induced abortion has also been associated with PGs **(Skarnes & Harper, 1972)**. In contrast, little is known of the expression pattern and function of the IP receptor in the human endometrium, although prostacyclin synthase and IP receptor expression have been demonstrated in pregnant and non-pregnant myometrium **(Giannoulias *et al.*, 2002).** However, to the best of our knowledge, none of the studies till date has elucidated the endometrial expression of PG contractile/ relaxatory gene receptors in the ECT of women having a history of spontaneous abortion and found to be harbouring *C. trachomatis* infection. The study demonstrated for the first time that a significant altered expression of

contractile/ relaxatory PG receptor genes in the ECT of *C. trachomatis*-positive SA and *C. trachomatis*-negative SA, thereby confirming the involvement of PG synthesis in abortion. Significantly more expression of PG receptors in the *C. trachomatis*-positive SA might be due to the *C. trachomatis* infection which led to an increase in COX-2 level in SA ultimately leading to an increased PG synthesis within the foetal membranes. No difference between the expressions of EP-1/ EP-2/ EP-3/ EP-4 was observed in the RSA/ SSA.

Also, a significant positive correlation was observed between advanced GA and contractile receptors, *viz.:* EP-1, EP-3 and FP, as evident by their increased mRNA expression. In humans, EP receptor mRNA expression may be temporally expressed with respect to gestation **(Leonhardt *et al.*, 2003)**. Few studies have also demonstrated a tendency towards decreased EP-2 receptor mRNA expression with advancing GA in humans **(Brodt-Eppley & Myatt, 1999)** and also in rat myometrium **(Leonhardt *et al.*, 2003),** while an increased EP-1 mRNA expression with advanced GA in baboon cervix was reported **(Smith *et al.*, 2001).** In our study, we found EP-1, EP-3 and FP receptors to be positively correlated with GA in *C. trachomatis*-positive spontaneous abortion however the expression of EP-2 and EP-4 was negatively correlated. Further research can focus on the control of expression of the EP-1, EP-3 and FP receptor genes in such women.

A well-developed placenta secretes adequate amounts of estrogen and progesterone. Both these hormones are responsible for maintaining the ovum during its early growth period. If their secretion is inadequate, early abortion may result. The endocrinology of infectious abortion has been the subject of intermittent attention

however this is restricted largely to animal studies. Studies revealed that progesterone as well as estrogen is capable of causing the release of PGs from the uterine tissue. Various animal model studies have reported that chlamydial LPS associated with adverse developmental outcomes **(Agarwal *et al.,* 2010).** It was reported that abortion in late gestation in ewes after infection with *C. psittaci* is probably due to an elevation of circulating estrogens and PGs and a drop of progesterone in maternal plasma **(Bosc *et al.,* 1981).** The early increase in plasma estrogen may also induce metabolic changes, which enhance chlamydial growth at a specific stage of late gestation **(Leaver *et al.,* 1989).** In primates, the decrease of progesterone reportedly causes labour **(Peltier, 2003).** *C. trachomatis* in trophoblast also showed a relative increase in protein of HSP-60 compared with MOMP, suggestive of chlamydial chronicity while the levels of estrogen and progesterone were decreased **(Azenabor *et al.,* 2007).** Changes in the concentrations of estrogen and progesterone influence the release of PGE2 at parturition **(Olson *et al.,* 1984).** However, till date, there is no clear consensus between PG production and hormone output in RSA. The available literature has focused largely on progesterone; an *in vitro* study concluded that decline in PG and rise in progesterone occur independently of each other **(Fowkes *et al*., 2001)** while others have reported negative regulation of PG receptors (PGE2/ PGF) by progesterone **(Ishihara *et al*., 1995).** In our study, an increased expression of PG receptors, low progesterone and increased estrogen levels was found in *C. trachomatis*-infected SA. It was further suggested that decreased progesterone increase uterine PG within the uterus **(Loose & Stancel, 2006)**.

Our results show that chlamydial infection results in an increase in various PG receptors and decreased progesterone level in women experiencing SA ultimately leading

to an upregulated PG synthesis within the foetal membranes. Our data further shows a positive correlation between few contractile gene receptors (EP-1, EP-3 and FP) and GA. Data suggests that increased expression of COX-2 and PG receptors, particularly contractile gene receptors, with advanced GA could be a possible risk factor for abortion in *C. trachomatis*-infected SA. *C. trachomatis* infection might have led to an increase in COX-2 level in SA ultimately leading to an increased PG synthesis within the foetal membranes. There is currently no information on the transcriptional regulation of PG receptor genes. An elucidation of the factors that control the expression of contractile EP-1, EP-3 and FP gene receptors may shed light on the underlying molecular pathway involved in spontaneous abortion among the *C. trachomatis*-infected women.

# CHAPTER 5

# EXPRESSION OF CYTOKINES IN SPONTANEOUS ABORTERS INFECTED WITH *CHLAMYDIA TRACHOMATIS*

## 5.1. Introduction

Spontaneous abortion is one of the most common complications of pregnancy and it occurs due to several identifiable causes such as anatomic, genetic, endocrinologic and infectious etiologies. Pregnancy also increases the risk of *Chlamydia trachomatis* colonization resulting in altered immune response and may affect intra- and extra-uterine development in pregnant women causing abortion **(Silva *et al.,* 2011).** The biphasic life cycle of *C. trachomatis* as well as its adaptation to evade the immune response allows organism to persist for extended periods within host epithelial cells, inducing a chronic inflammatory response **(Rasmussen *et al.,* 1997; Kessler *et al.,* 2012).** It was demonstrated that once *C. trachomatis* has established infection within epithelial cells, the innate immune response allows for the production of pro-inflammatory cytokines such as interleukins (IL) (IL-1, IL-6, IL-8), and TNF-α **(Rasmussen *et al.,* 1997).**

Previous studies have investigated the inflammatory response of *C. trachomatis* in the initial stages of infection, including regulation by cytokines, chemokines and inflammatory mediators involved in the recruitment of immune cells **(Kessler *et al.,* 2012; Igietseme *et al.,* 2013)**. *C. trachomatis* also induces production of TNF-α, which promotes apoptosis of infected and bystander cells **(Darville *et al.,* 2003).** Upregulated cytokines expression (TNF-α, IFN-γ and IL-12) was also found in the cervical secretions of *C. trachomatis* infected women **(Reddy *et al.,* 2004).** Other study demonstrated in mice that the concurrent expression of mRNA encoding multiple cytokines (TNF-α, IFN-γ, IL-8 etc.) in spatially distinct sections of reproductive tract like vaginal and uterine tissues **(Cerny *et al.,* 2015).** Furthermore it was found out that the synergistic effect of IFN-γ and TNF-α play a role in host defence against infection and in the establishment of

persistent chlamydial infection inside the host **(Ishihara *et al.,* 2005).** In response to infection, there is an abnormal production of proinflammatory cytokines occur in the placenta **(El-Shazly *et al.,* 2004)**.

The immune system within the female genital tract faces the unique challenge of protecting the host against infectious pathogens while being tolerant to the local beneficial microbiota and to an immunologically distinct fetus. The immunologic cytokines may facilitate or hinder pregnancy, depending on the type of cytokine present. It has been reported that many miscarriages are caused by abnormal levels of cytokines to control the inflammatory process in the body. Early exposure to proinflammatory cytokines is necessary in stimulating invasion of the blastocyst and the formation of new blood vessels during implantation. However, if the exposure to proinflammatory cytokines is prolonged, it can actually be severely detrimental to the pregnancy and result in a loss of the fetus. Cytokines also play a fundamental role in pregnancy outcome **(Van Bodegom *et al.,* 2007)**. Maternal tolerance toward fetal alloantigens was explained by the predominant Th2 type immunity during pregnancy, which overrules Th1 type immunity, therefore protecting the fetus from maternal Th1 cell attack **(Wegmann *et al.,* 1993).** Indeed, predominant Th1 type immunity has been observed in recurrent spontaneous abortion **(Raghupathy, 1997)**. In the animal model, the immunological profile of early pregnancy loss is biased towards an excessive Th1 cytokine profile **(Tangri *et al.*, 1994).** Partial systemic impairment of Th1 responses is compatible with clinical evidence that a number of infectious diseases caused by intracellular pathogens can sometimes be exacerbated in pregnancy, e.g. cytomegalovirus and malaria **(Hart, 1988).** An abnormal Th1 type cellular immune response is the basis for immunological reproductive failure in

women **(Hill *et al.,* 1995).** Peripheral blood mononuclear cells (PBMC) from women with recurrent pregnancy loss (RPL) respond to trophoblast extracts *in vitro* by releasing Th1 type cytokines (TNF-α and IFN-γ) and a reduction in Th2-type cytokine production (IL-4 and IL-10) **(Raghupathy *et al.*, 1999).**

In humans, TNF-α is also known to inhibit trophoblast proliferation, which may be of clinical importance in implantation and early pregnancy when trophoblast invades the endometrium **(Hunt *et al.*, 1990).** In mammals deregulated expression of cytokines and their signaling leads to an absolute or partial failure of implantation and abnormal placental formation **(Guzeloglu-Kayisli *et al.,* 2009).** Circulating levels of TNF-α and IFN- γ are higher in patients with a subsequent miscarriage compared with those with a successful pregnancy, suggesting that these cytokines may also be a potentially relevant factor in RPL patients. An aberrant expression of proinflammatory cytokines was found in women with idiopathic recurrent spontaneous miscarriage **(Banerjee *et al.,* 2013).** Also, a higher expression of proinflammatory cytokines is found in recurrent miscarriage **(Daher *et al.,* 2004).** In addition, the cervical IL-8 concentrations have also been reported to be associated with vaginal bacteria in pregnancy and intrauterine infections in patients with pre-term labor, providing an indication for treatment to prevent pre-term birth **(Sakai *et al.,* 2004).**

Furthermore, inflammatory cytokines have been reported to induce transforming growth factor-β (TGF-β), a multifunctional cytokine with a wide range of physiologic and pathologic effects **(Matsumura *et al.,* 2009).** TGF-β is a member of the family of growth factor cytokines, with five different isoforms. TGF-β binds to at least eight different types of receptor, each with different properties. It is produced by almost all

nucleated cells functioning as a growth factor as well as promoting cell differentiation, proliferation and morphogenesis **(Tabibzadeh, 1991).** Within the immune system, TGF-β promotes the generation of Th-1 cells. In the animal model, TGF-β mRNA has been shown by in-situ hybridization to be expressed in endometrium and decidua during the preimplantation period and TGF-β gene expression has been localized using immunohistochemical staining to endometrial and stromal cells on days 1 to 4 of murine pregnancy **(Tamada *et al.*, 1990).** In the endometrium TGF-β modulates epithelial proliferation, enhances gland formation, promote angiogenesis and deposition of extracellular matrices such as collagen **(Quaglino *et al.*, 1990).** TGFs are expressed during most of gestation in human and murine pregnancy. TGF-β1 has been shown to be the key factor that controls trophoblast growth as well as trophoblast invasion of the uterus *in situ*. TGF-β1 exists in the trophoblast and may inhibit placental differentiation and human chorionic gonadotropin directly **(Graham *et al.,* 1992; Song *et al.,* 1996).** TGF-β1 is important for regulation of cytokine network during pregnancy **(Graham *et al.,* 1992)** and plays a role in regulating maternal rejection of fetoplacental unit and preventing miscarriages. TGF-β2 is synthesized by maternal decidual cells and uterine epithelium **(Cheng *et al.,* 1993).** The expression of TGF-β2 appears to be regulated by pregnancy hormones.

It is apparent that till date, the immunological changes underlying the complex transition from uterine quiescence to spontaneous abortions are not clear in *C. trachomatis*-infected women. We hypothesized that altered level of pro-inflammatory cytokines might be involved in *C. trachomatis*-infected spontaneous abortion further triggering the COX-derived prostaglandins signalling pathway leading to abortion in

women. Hence, the study aimed to quantitate the expression of proinflammatory cytokines, *viz.:* TNF-α, IFN-γ and IL-8 and multifunctional cytokines TGF-β1, TGF-β2 in the endometrial curettage tissues (ECT) of *C. trachomatis*-infected spontaneous aborters (SA) and attempted to find whether there was any correlation between cytokines and Cox-2 in *C. trachomatis*-positive SA.

## 5.2. Results

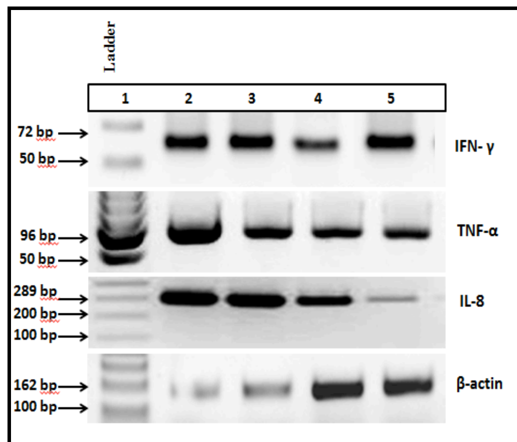
### 5.2.1. Qualitative analysis of mRNA expression for proinflammatory cytokines (TNF-α, IFN-γ, IL-8) and multifunctional cytokines (TGF-β1, TGF-β2) by reverse transcription PCR

Expression of TNF-α, IFN-γ, IL-8 genes in the ECT was checked by performing RT- PCR in relation to the housekeeping gene, *viz.:* beta actin in *C. trachomatis*-positive patients in study group in comparison to the controls by using reverse transcription PCR (RT-PCR) assay. In all *C. trachomatis*-positive endometrial tissue specimens, clear signals were detected for the cDNA amplification products of the TNF-α, IFN-γ and IL-8 **(Fig. 5.1).** The expression of these pro-inflammatory cytokines was found significantly higher in *C. trachomatis*-positive SA in comparison to the *C. trachomatis*-negative control group (‘*p’* $< 0.0001$; arbitrary units; **Fig. 5.2**).

Further, the mRNA expression of TGF-β genes (TGF-β1/ TGF-β2) was elucidated in the ECT of *C. trachomatis*-positive SA by using RT- PCR. Housekeeping gene β-actin was used as an internal control. In all *C. trachomatis*-positive endometrial tissue

specimens, clear signals were detected for the cDNA amplification products of the TGF-β1/ TGF-β2 **(Fig. 5.3).** The expression of TGF-β1 and TGF-β2 was significantly high in *C. trachomatis*-positive SA in comparison to the control group ($p$ < 0.0001; arbitrary units; **Fig. 5.4**).

**(5.1)** **(5.2)**



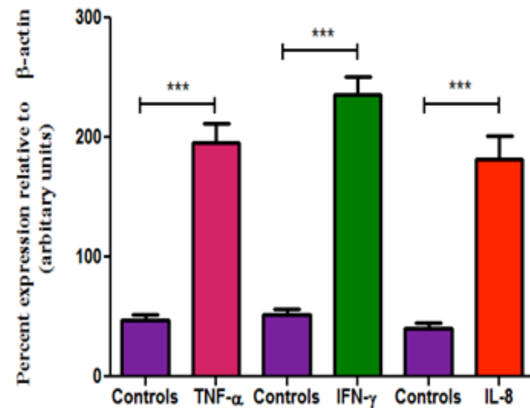




**Fig. 5.1:** Amplification products for proinflammatory cytokines (TNF-α, IFN-γ, IL-8) in the ECT of spontaneous aborters. Lane - 1 shows 50/100 bp ladder, lanes 2 - 5 show cytokines expression in ECT of *Chlamydia trachomatis*-positive samples.

**Fig 5.2:** Increased expression of TNF-α, IFN-γ, IL-8 genes in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group by RT-PCR (Non-parametric Mann-Whitney test; '$p$'< 0.0001).

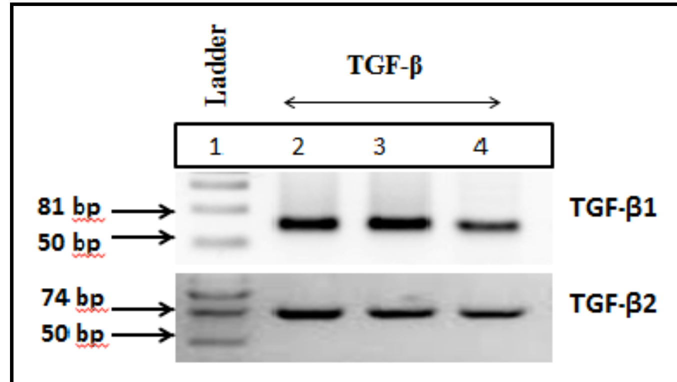



**Fig 5.3:** Amplification products of multifunctional cytokine TGF-β in ECT of spontaneous aborters; Lane - 1shows 50 bp ladder, lanes 2 - 5 show TGF-β1/ TGF-β2 expression in the ECT of *Chlamydia trachomatis*-positive samples

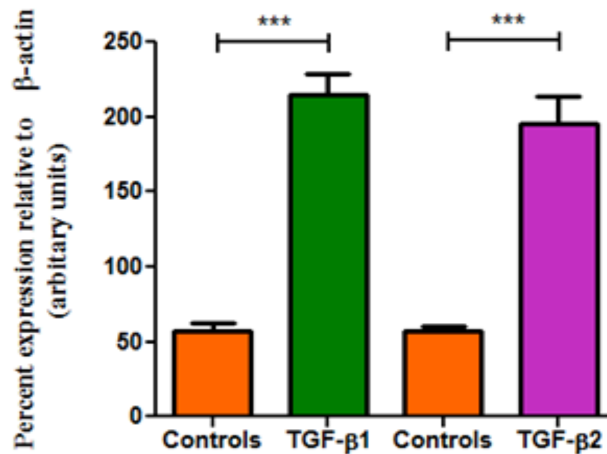



**Fig 5.4:** Expression of TGF-β1/ TGF-β2 genes in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group by RT-PCR (Non-parametric Mann-Whitney test; '$p$' $< 0.0001$)

### 5.2.2. Quantitative analysis of mRNA expression for proinflammatory cytokines (TNF-α, IFN-γ, IL-8) and multifunctional cytokine (TGF-β1/ TGF-β1) by real time PCR

To determine whether altered mRNA expression of cytokines was associated with spontaneous abortion, expression of mRNAs encoding cytokines was quantified in endometrial biopsies (ECT) by quantitative real time PCR (q–PCR). The q–PCR assays used in this study were not designed to allow quantitative comparisons of mRNA abundance between different cytokines. However, by comparing mean Ct values for control endometrial biopsies, it is possible to gain a qualitative estimate of relative cytokine expression levels.

At transcript level, the analysis of mRNA expression for TNF-α, IFN-γ and IL-8 genes within the ECT was compared among patient group as a ratio to the expression of constitutively expressed GAPDH and β-actin gene by performing quantitative real time PCR. The abundance of mRNAs expression of Th-1 cytokines TNF-α, IFN-γ and IL-8 mRNA demonstrated a significant increase in the endometrial curettage tissue of *C. trachomatis*-positive SA (Group I) in comparison to Group II control women. The expression of these cytokines was significantly high in *C. trachomatis*-positive SA versus *C. trachomatis*-negative SA (Non-parametric Mann-Whitney test, **'*p*' < 0.002; **Fig. 5.5 a-c**). TNF-α, IFN-γ and IL-8 cytokines expression was also significantly high in *C. trachomatis*-negative SA versus controls (Non-parametric Mann-Whitney test, ***'*p*' < 0.0001; **Fig. 5.5 a-c**). Upon analysis of Ct values, the TNF-α gene was found 3-fold upregulated in *C. trachomatis*-infected women undergoing spontaneous abortion, however the IFN-γ gene was found to be 4.5- fold upregulated and 2- fold upregulation

was found in IL-8 cytokine **(Table 5.1).** The mRNA expression of TNF-α, IFN-γ and IL-8 genes was also compared between the RSA and SSA sub groups and it was found that TNF-α, IFN-γ IL-8 genes expression was significantly higher in the RSA sub group in comparison to the SSA (Mann-Whitney test, '*p*' < 0.002; **Fig. 5.6 a-c**).

**Table 5.1:** Expression of TNF-α, IFN-γ, IL-8, TGF-β1 and TGF-β2 genes in the ECT; Fold change values in gene expression are presented as average fold change ($2^{-(\text{average Ct})}$) for differentially expressed mRNAs ('*p*' < 0.05).

| Cytokine | Spontaneous aborters Avg. ΔCt | Controls Avg. ΔCt | Fold Change | '*p*'- value |
|---|---|---|---|---|
| **TNF-α** | 6.36 | 7.93 | 3.0 | < 0.0001 |
| **IFN-γ** | 8.32 | 10.6 | 4.5 | < 0.0001 |
| **IL-8** | 6.83 | 7.82 | 2.0 | < 0.0001 |
| **TGF-β1** | 7.42 | 8.41 | 2.0 | < 0.0001 |
| **TGF-β2** | 7.50 | 8.63 | 2.2 | < 0.0001 |

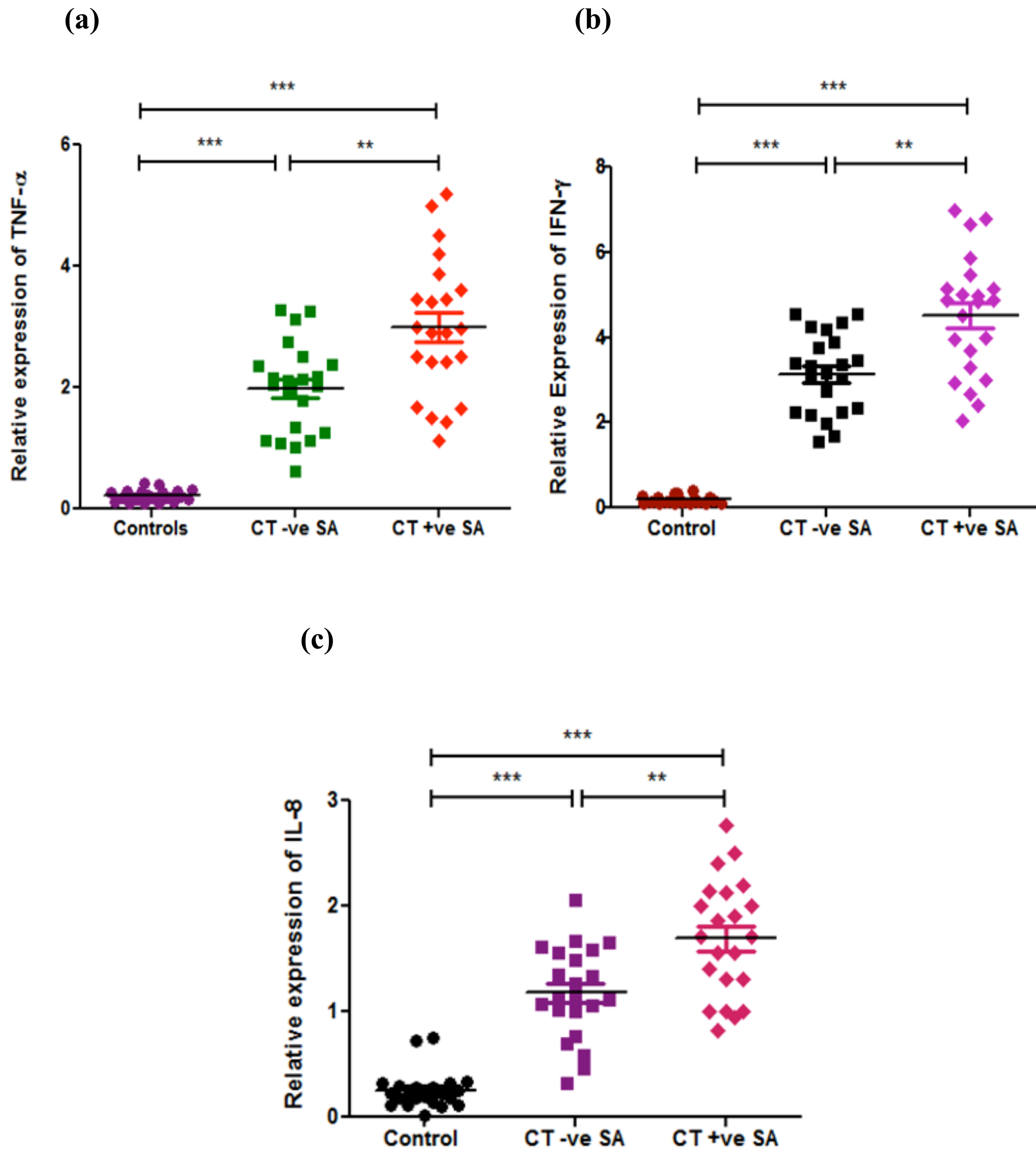



**Fig. 5.5: (a-c)** Expression of TNF-α/ IFN-γ/ IL-8 genes in *Chlamydia trachomatis*-positive spontaneous aborters in comparison to *Chlamydia trachomatis*-negative spontaneous aborters and controls (Non-parametric Mann-Whitney test; ***'*p*' $< 0.0001$) ('*p'* value *** = $< 0.0001$; ** = $< 0.001$). **Abbreviations:** SA – spontaneous aborters; CT – *Chlamydia trachomatis.*

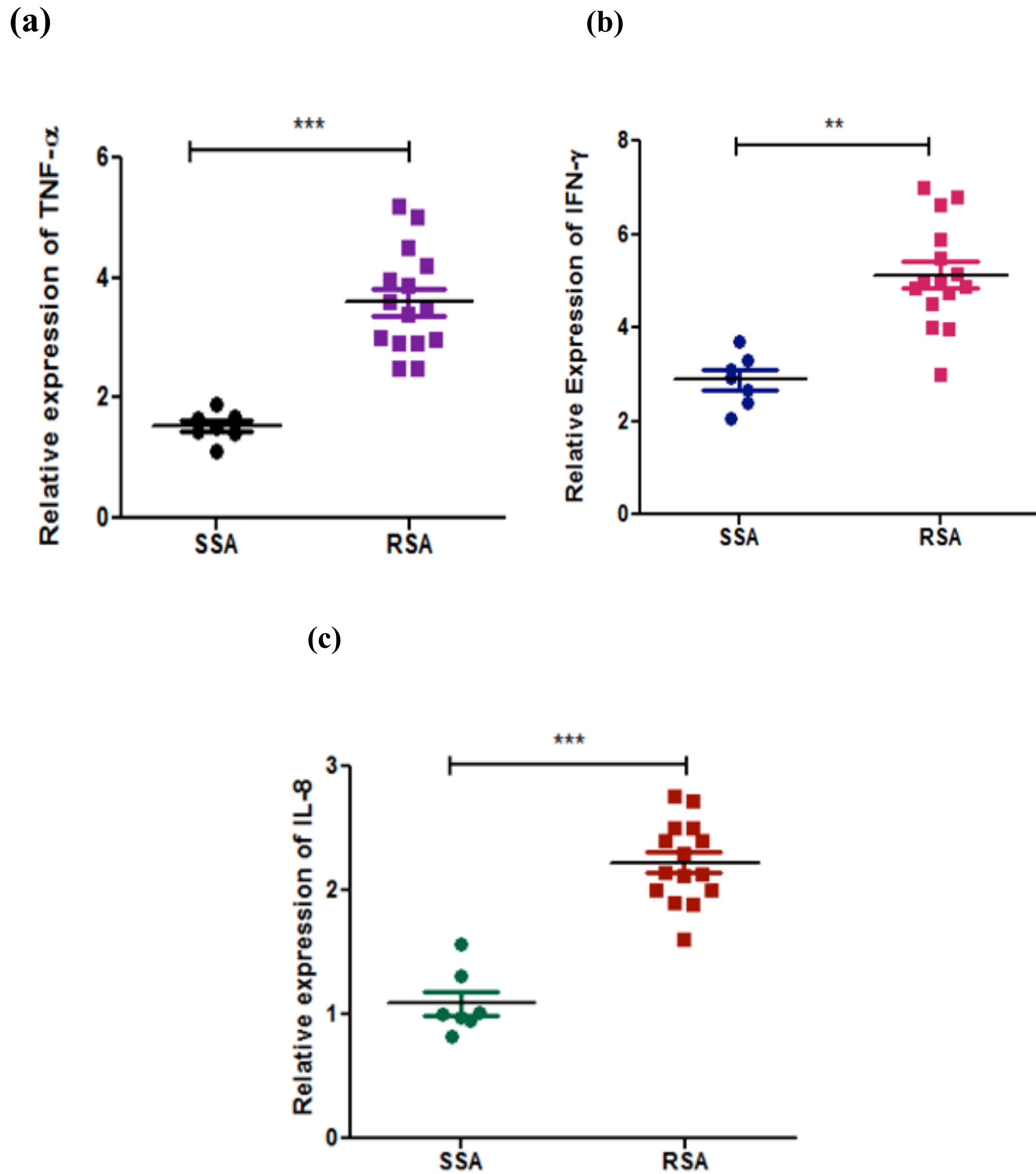



**Fig 5.6: (a-c)** Expression of TNF-α/ IFN-γ/ IL-8 was significantly high in RSA (Sub-group Ib) versus SSA (Sub-group Ia) (Non- parametric Mann-Whitney test; ***'*p*'< 0.0001) ('*p*' value *** = < 0.0001; ** = < 0.001). **Abbreviations:** SA – spontaneous aborters; SSA – sporadic spontaneous aborters; RSA – recurrent spontaneous aborters

The multifunctional cytokine associated with T-regulatory cell differentiation is TGF-β, and mRNAs encoding each of the two TGF-β isoforms TGF-β1and TGF-β2 were examined in the ECT of aborters. TGF-β1 and TGF-β2 expressed significantly at similar levels in the ECT of *C. trachomatis*-positive SA (Group I) in comparison to Group II control women (Non- parametric Mann-Whitney test; ***'*p*' < 0.0001), however there was no significant difference observed in the expression of TGF-β1 and TGF-β2 between *C. trachomatis*-positive SA and *C. trachomatis*-negative SA (Non- parametric Mann-Whitney test; # '*p*' > 0.05 **(Fig. 5.7 a-b)**. Also, no significant difference was observed in the expression of TGF-β1 and TGF-β2 in RSA (Sub-group Ib) versus SSA (Sub-group Ia) (Non- parametric Mann-Whitney test; #'*p*'> 0.05) **(Fig. 5.8 a-b).**

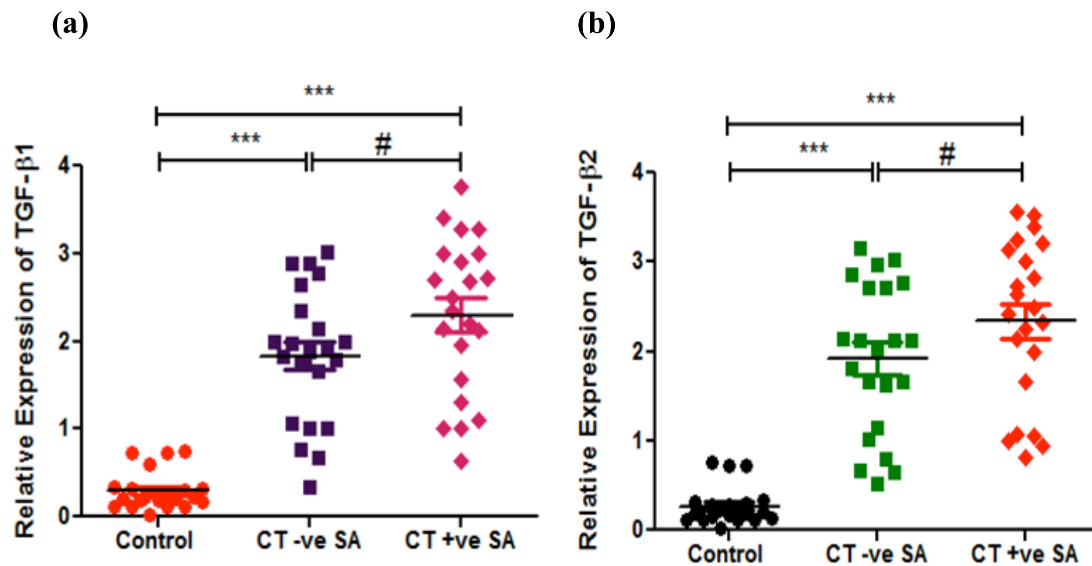



**Fig 5.7: (a-b)** Expression of TGF-β1/ TGF-β2 gene was significantly high in *Chlamydia trachomatis* positive spontaneous aborters (Group I) (Non-parametric Mann-Whitney test; ***'*p*'< 0.0001) ('*p*' value *** = < 0.0001; ** = < 0.001, # = non-significant). **Abbreviations:** CT – *Chlamydia trachomatis*; SA – spontaneous aborters.

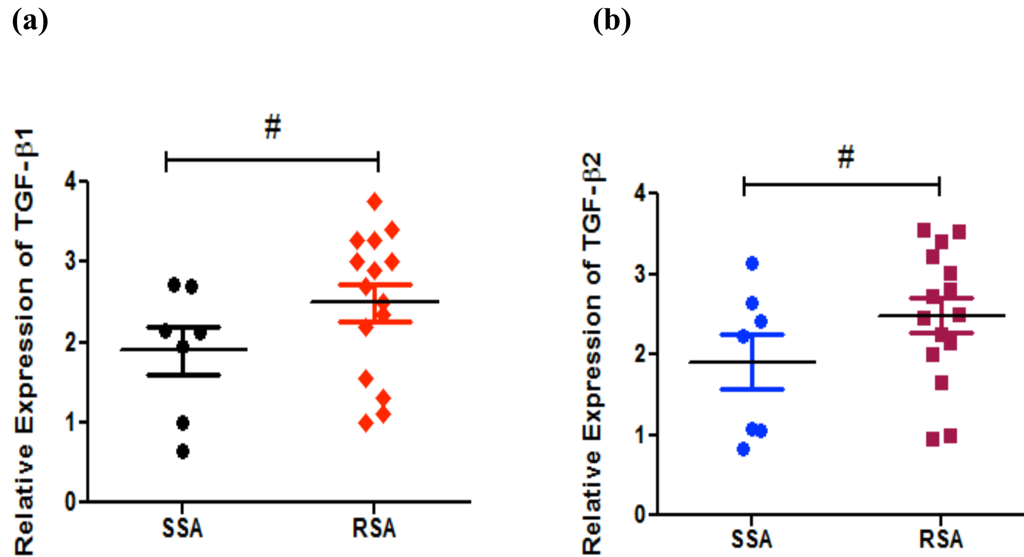



**Fig. 5.8: (a-b)** Expression of TGF-β1 and TGF-β2 was not significant in RSA (Sub-group Ib) versus SSA (Sub-group Ia) (Non- parametric Mann-Whitney test; (# = non-significant).

### 5.2.3. Correlation between cyclooxygenase-2 and cytokines in spontaneous aborters infected with *Chlamydia trachomatis*

A correlation was made among the cytokines and COX-2 in the *C. trachomatis*-positive SA to check whether the cytokines induces the production of COX-2, which further triggers the PG signalling pathways leading to abortion. A statistically significant positive correlation was found in the COX-2 and TNF-α (Spearman rank correlation; r = 0.87, 95% CI; 0.70- 0.94; ***‘*p*’ < 0.0001, **Fig. 5.9 a**). However, COX-2 and IFN-γ were not found significantly correlated in infected aborters (Spearman rank correlation; r = 0.38, 95% CI; - 0.70- 0.07; #‘*p*’: 0.15, **Fig. 5.9 b**). The IL-8 expression in ECT was also

insignificant in correlation with COX-2 (Spearman rank correlation; r = 0.28, 95% CI; -0.178- 0.65; #'*p*': 0.22, **Fig. 5.9 c**).

TGF-β1/ TGF-β2 and COX-2 expression in ECT from women infected with C. trachomatis and undergoing spontaneous abortion was positively correlated with each other, which means, that a higher TGF-β1/ TGF-β2 expression was accompanied by higher COX-2 expression (Spearman rank correlation; TGF-β1: r = 0.70, 95% CI; 0.37- 0.87; **'*p*' < 0.002; TGF-β2: r = 0.70, 95% CI; 0.28- 0.85; *'*p*' < 0.05, **Fig. 5.9 d-e**). In the study group we have been able to show a correlation between expression of TGF-β1 and COX-2, and between COX-2 and TGF-β2.

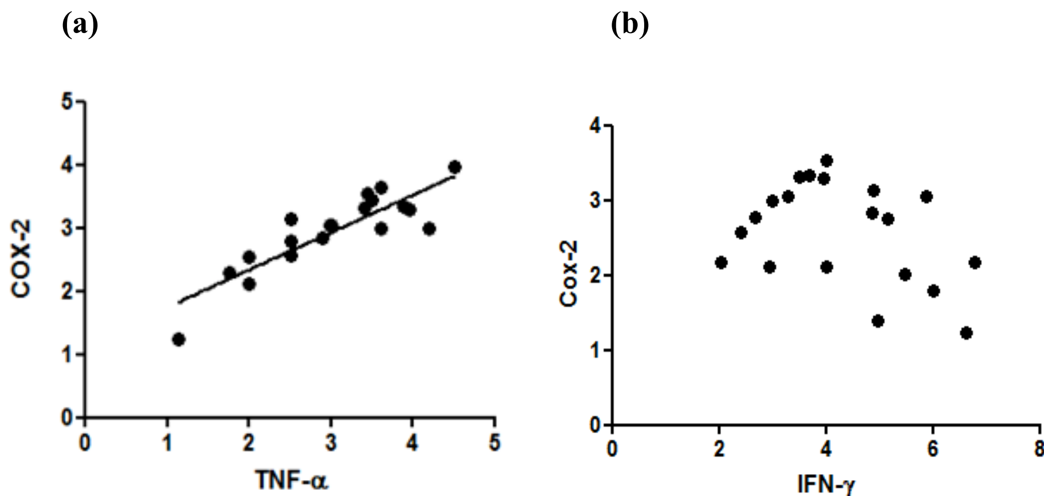



**Fig. 5.9: (a- b)** Determination of correlation between COX-2 and cytokines with spearman rank correlation test

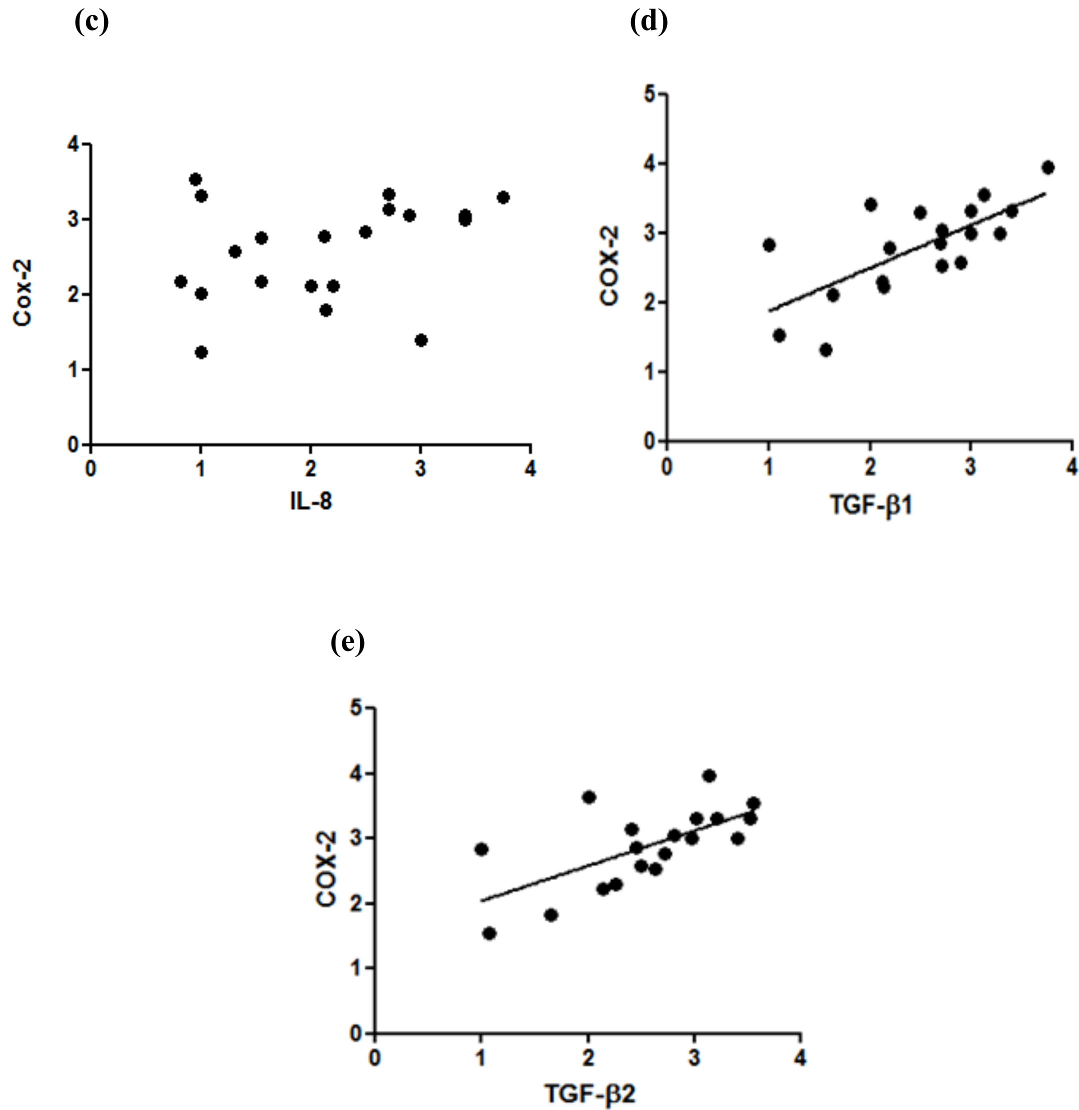



**Fig. 5.9: (c- e)** Determination of correlation between COX-2 and cytokines with spearman rank correlation test

### 5.2.4. Discussion

Pregnancy is often referred, as an "immunological paradox" because tissue allografts are generally rejected by an immunocompetent host, while the conceptus, the so-called foetal allograft, is not rejected by the maternal immune system. The success of the foetus in the face of a potentially hostile maternal immune system has been suggested

to be due to immunomodulation at the maternal-foetal interface **(Chaouat *et al.,* 1997)** and a consequent lack of strong maternal cell-mediated anti-foetal reactivity of the Thl type. *In vivo* and *in vitro* studies in the mouse and *in vitro* studies in the human system have shown that a strong, maternal Thl type reactivity is deleterious to pregnancy **(Raghupathy *et al.,* 1997).**

The causes of pregnancy loss are not well understood, but infection and immune mechanisms are thought to be involved. The complex interplay between maternal and foetal immune mechanisms changes temporally as pregnancy progresses **(Tranchot-Diallo *et al.*, 1997).** If this delicate balance is adversely affected, immunoregulatory mechanisms may be insufficient to restore homeostasis and this may lead to pregnancy failure. It has been proposed that during pregnancy, systemic maternal immune responses are biased in favour of a Th2 cytokine profile **(Wegmann *et al.,* 1993).** Successful pregnancy may depend, at least in part, on the bias of the maternal immune response shifting away from Th1 type responses towards a Th2 phenotype, both in murine models and humans. Attention has focused on elucidating the immunobiological role of cytokines in normal human pregnancy following the accumulated reports of complex cytokines activity within uteroplacental tissues **(Robertson *et al.,* 1994).** Th cells can differentiate into subsets with distinctive patterns of cytokine release. It has been proposed that Th1 type responses (production of IL-2 and IFN-γ) are systemically suppressed in murine pregnancy **(Wegmann *et al.*, 1993).** TNF-α is an important immunoregulatory cytokine, which may be produced in Th1 or Th2 type responses and is known to have different effects depending on gestational age. It has been shown that TNF-α production by PBMC is suppressed at the mRNA level during early pregnancy and a significant increase does

not occur until the eighth month of gestation **(Tranchot-Diallo *et al.*, 1997).** TNF-α production in late pregnancy is implicated in the induction of labour in humans **(Vince *et al.*, 1992).** Significant levels of the Th1 cytokines, IFN-γ and IL-2 have been detected at the maternal–foetal junction in cases of spontaneously aborting conceptus **(Tangri *et al.*, 1993).**

Our study showed a statistically significant increased expression of all pro-inflammatory cytokines, *viz.:* TNF-α, IFN-γ and IL-8 in *C. trachomatis*-positive SA in comparison to the *C. trachomatis*-negative SA and uninfected controls. This might be because of the *C. trachomatis* infection. In response to infection, an abnormal production of proinflammatory cytokines, such as IL-1β, TNF-α, IL-6, and IL-8 was found in the placenta **(El-Shazly *et al.*, 2004),** which may eventually lead to spontaneous abortion. Our results further revealed a high expression of TNF-α, IFN-γ and IL-8 cytokines in the RSA sub-group in comparison to the SSA. Higher TNF-α, IFN-γ production in RPL patients at the time of miscarriage compared to healthy pregnant women has been reported **(Makhseed *et al.,* 2000).** These study results are in line with our results. In another study, TNF-α was found abundantly in the decidua in the RPL women who had miscarried **(Vives *et al.*, 1999).** Further, elevated values of TNF-α and IFN-γ were reported in all 100% cases in the recurrent miscarriage group while none of control women had elevation of TNF-α or IFN-γ **(Hill *et al.,* 1995).** The central role of TNF-α in pregnancy may be important in determining the outcome of pregnancy in RPL women whose immunoregulatory network may be compromised before pregnancy occurs. Higher concentrations of Th1 cytokines (TNF-α) have been demonstrated systemically in the serum of patients with idiopathic/unexplained recurrent miscarriages than normal

controls **(Mallmann *et al.*, 1991).** Women with RPL were found to have a Th1 cytokine profile in peri-implantation endometrium however, this profile did not predict pregnancy outcome **(Lim *et al.*, 2000).** In another study, increased levels of IL-8 in amniotic fluid have been found in cases of preterm labour with histologically confirmed chorioamnionitis **(Lockwood *et al.,* 2004).** The increased level of IL-6 and IL-8 were also observed in women with second trimester abortions **(Galazios *et al.,* 2011).** Recently, a relationship between both IL-6 and IL-8 in cervical and amniotic fluid and microbial invasion of the chorioamniotic membranes was also reported **(Jacobsson *et al.,* 2005).**

Furthermore, TGF-β is likely to contribute to placental function and fetal development **(Ingman & Robertson, 2002).** The level of TGF-β1 levels rise during pregnancy in normal case **(Ogasawara *et al.,* 2000).** The present study indicated that the excessive production of TGF-β1and TGF-β2 might be associated with spontaneous abortion in *C. trachomatis* infected women. TGF-β1 has been shown to be the key factor that controls trophoblast growth as well as trophoblast invasion of the uterus *in situ* and it was found that lymphocyte populations secreting TGF-β are causally linked with pregnancy success in mice and are diminished in the event of miscarriage **(Arck *et al.,* 1999).** It has been further suggested that TGF-β1 is necessary for pregnancy development but may also represent a risk factor for recurrent abortions (**Ogasawara *et al.,* 2000**). In our study, TGF-β1 and TGF-β2 expressed significantly at almost similar levels in the ECT of *C. trachomatis*-positive SA (Group I) in comparison to Group II control women; however there was no significant difference observed in the expression of TGF-β1/ TGF-β2 in the *C. trachomatis*-positive SA versus *C. trachomatis*-negative SA. Also, no

significant difference observed in the expression of TGF-β1 and TGF-β2 in RSA versus SSA.

A statistically significant positive correlation was also found between TNF-α and COX-2 in the *C. trachomatis* positive SA. In a murine model, it was found that abnormal expression of COX-2, TNF-α and IL-6 resulted in recurrent spontaneous abortion **(Hua *et al.*, 2013).** COX-2 is an inducible enzyme which is expressed upon bacterial product stimulation such as LPS or immunological stimuli including IL-1 or TNF-α **(Herschman et al., 1996)** and a higher expression of COX-2 during the implantation window in the endometrial tissue of women with idiopathic recurrent spontaneous miscarriage may be attributed to the exaggerated expression of proinflammatory cytokines such as TNF-α and IFN-γ **(Banerjee *et al.,* 2013).** IL-6 and TNF-α can induce the expression of COX-2 and then causing increased PGs. Therefore, when the expression of cytokines like IL-6 and TNF-α were in the normal level, the COX-2 can form the dynamic equilibrium, which can ensure the safe implantation of embryo sac and avoid abortion **(Yu *et al.,* 2010; Zhang *et al.,* 2010; Parveen *et al.,* 2013).**

Furthermore, in our results, we found a non-significant correlation between COX-2 and IFN-γ. The non-significance between COX-2 and IFN-γ is because the expression of IFN-γ was quite high in comparison to COX-2. IFN-γ levels were found significantly higher in cervical washes of women with recurrent infection **(Agarwal *et al.,* 2007).** IFN-γ is also known for its requirement in resolution of chlamydial infections, and has been shown to inhibit the growth of *Chlamydia* in cell culture **(Rottenberg *et al.,* 2002).** The continued high expression of the IFN-γ can lead to the increase and the aggregation of the inflammatory cytokine concentration, and the interaction between cells can also cause

cytotoxic effect, which lead to the imbalance of immune tolerance and then induced SA. IFN-γ can induce the expression of COX-2 and then causing increased PGs, which can further lead to SA. TGFβ1/ TGFβ2 and COX-2 expression in ECT from women infected with *C. trachomatis* and undergoing spontaneous abortion was also found positively correlated with each other, which means, that a higher TGFβ1/ TGFβ2 expression was accompanied by higher COX-2 expression. In the study group, we have been able to found a positive correlation between expression of TGF-β1/ TGF-β2 and COX-2.

Apparently, spontaneous abortion is a pleiotropic condition with several as yet unidentified causes; however an aberration in cytokine production also constitutes an important contributing factor in *C. trachomatis*-positive SA. Our data support the hypothesis of Th1 cytokine involvement in the pathogenesis of SA. It may be concluded that modulation of Th1 dominance may bring about an immunological milieu that is more conducive to successful pregnancy. An overall understanding of cytokine production in SA during *C. trachomatis* infection shall contribute to the development of better management and therapeutic intervention for a successful pregnancy. Further, anti-inflammatory drugs such as COX-2 inhibitors might be effective for prevention. It acts against non-specific inflammatory changes, and the IFN-γ, TNF-α and IL-8 might thus be important targets.

# CHAPTER 6

# EXPRESSION OF MATRIX METALLOPROTEINASES/ INHIBITORS IN *CHLAMYDIA TRACHOMATIS*-INFECTED SPONTANEOUS ABORTERS

## 6.1. Introduction

The mechanisms of *Chlamydia trachomatis* disease pathology are not completely understood. Some evidence suggests that the dysregulated extra cellular matrix (ECM) proteolysis seen during the processes of tissue repair following infection and inflammation **(Abu el-Asrar *et al.,* 2001)** may play a key role in the development of fibrotic sequelae of chlamydial infection in humans. MMPs are a class of zinc-dependent enzymes that are involved in the proteolysis and re-synthesis of the ECM **(Visse & Nagase, 2003)**, processing of chemokines and cytokines to active forms **(Opdenakker *et al.,* 2001),** the release of sequestered growth and signaling factors and chemotaxis and migration of leukocytes through inflamed tissues **(Maitra *et al.,* 2005)**. With regard to chlamydial infections, a role for MMP has also been proposed in trachoma **(Abu el-Asrar *et al.,* 2000; 2001)** and enhanced MMP expression has been reported in an *in vitro* model of human fallopian tube infection **(Ault *et al.,* 2002).** Inhibition of MMP by the administration of chemical inhibitors of their activity was shown to reduce inflammation, ascension of the infection into the upper genital tract and reduce hydrosalpinx formation **(Imtiaz *et al.,* 2006).** It was further shown that ocular *C. trachomatis* infection upregulates the expression of MMP-9 in the human conjunctival epithelium **(Burton *et al.,* 2004).** In addition, recent comparative studies of the role of MMP-9 in genital *Chlamydia muridarum* infection found greater MMP-9 activity during infection in those mouse strains exhibiting increased susceptibility to fibrotic sequelae following infection **(Ramsey *et al.,* 2005).**

In the female reproductive tract, MMPs play an important role in normal ovulation, menstruation, implantation, and placental development **(Fata *et al.,* 2000).**

However, in certain gynecologic and obstetric diseases, MMPs have been shown to play a pathogenic role. MMP-9 levels were found to be elevated in the amniotic fluid of women with preterm premature rupture of membranes **(Athayde *et al.,* 1998).** MMP-2 and MMP-9 are found involved in successful cytotrophoblast invasion in early pregnancy **(Moore & Crocker, 2012).** MMP-9 is the major protease involved in the invasion of human cytotrophoblast cells into the endometrium, and plays a major role in the process of embryo implantation and development **(Whiteside *et al.,* 2001).** MMP-2 and MMP-9 are the main mediators of endometrial ECM turnover during menstruation **(Kim *et al.,* 2004).**

MMP family members are inhibited by endogenous tissue inhibitors namely tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs and TIMPs both appear to play some roles in embryo implantation, trophoblast invasion, early placentation, and cervical dilatation and feto-maternal membrane lysis in later gestation. Serum TIMP-2 levels were found higher in the patients of recurrent pregnancy loss **(Anumba *et al.,* 2010).** There have been an increasing number of studies concerning the roles of MMPs and TIMPs in the abortion process. A higher ratio of MMP-9/TIMP-1 was found in IRSM suggestive of excessive degradation of the ECM **(Banerjee *et al.,* 2013).**

Despite a plethora of literature describing the role of MMPs/ TIMPs in pregnancy and miscarriage, there is a paucity of data on the *C. trachomatis* infection induced MMPs/ TIMPs leading to spontaneous abortion hence the present study was undertaken to elucidate the expression of MMP-2/ MMP-9 and TIMP-1/ TIMP-3 in *C. trachomatis* infected spontaneous aborters (SA) and to correlate their expression with the TGF-β and COX-2 expressions.

## 6.2. Results

### 6.2.1. Qualitative expression of matrix metalloproteinases (MMP-2/ MMP-9) and tissue inhibitors of metalloproteinases (TIMP-1/ TIMP-3)

Expression of MMP-2/ MMP-9 genes in the ECT was checked by performing RT-PCR in relation to the housekeeping gene, *viz.:* beta actin in *C. trachomatis*-positive patients in study group in comparison to the controls by using reverse transcription PCR (RT-PCR) assay. In all *C. trachomatis*-positive endometrial tissue specimens, clear signals were detected for the cDNA amplification products of the MMP-2 and MMP-9 genes **(Fig. 6.1 a, b).** The MMP-2 and MMP-9 expression was found significantly higher in *C. trachomatis*-positive SAs in comparison to the *C. trachomatis*-negative control group, ($p<0.0001$; arbitrary units; **Fig. 6.2 a, b).**

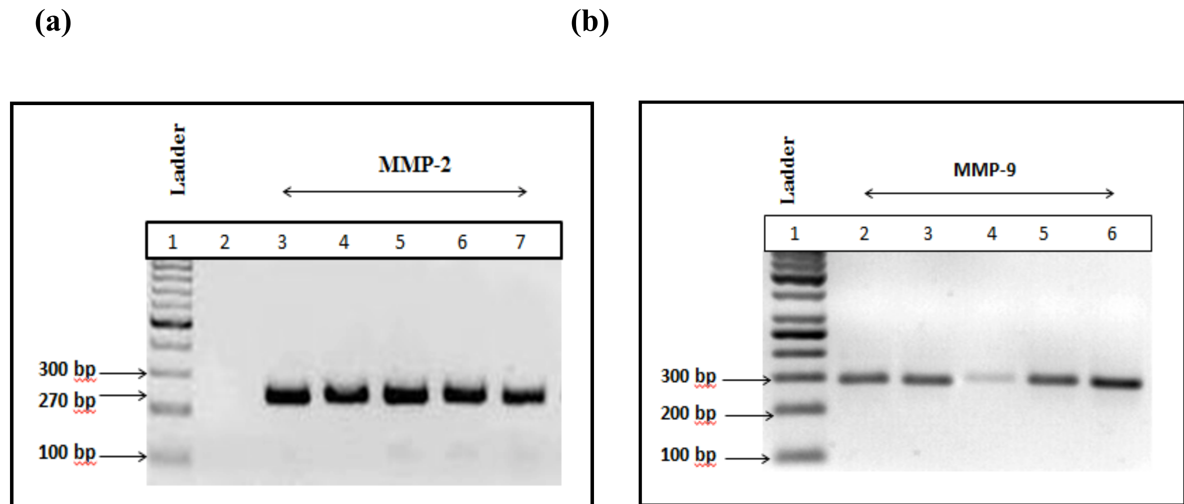



**Fig. 6.1 a, b:** Amplification products for MMPs in ECT of spontaneous aborters. (a) is showing the amplification products of MMP-2. (b) shows the amplification products of MMP-9; Lane- 1shows 100 bp ladder, lanes 2 - 6 show MMP-2 and MMP-9 expression in the ECT of *Chlamydia trachomatis*-positive samples.

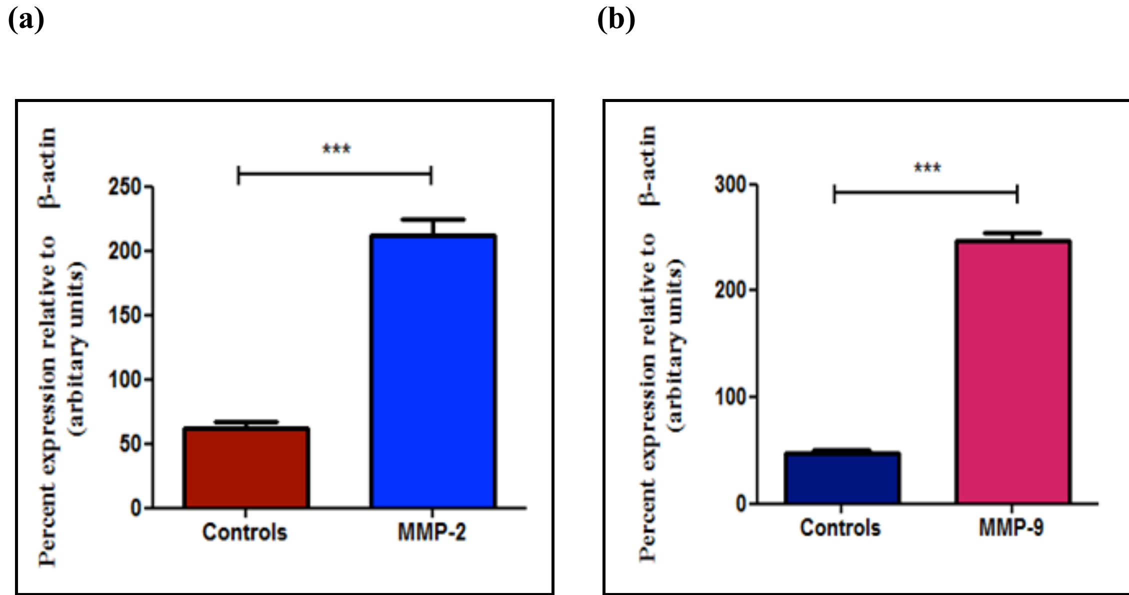



**Fig. 6.2 a, b:** Expression of MMP-2 and MMP-9 genes in *Chlamydia trachomatis* positive spontaneous aborters (n=22) *versus* control group by RT-PCR (Non-parametric Mann-Whitney test '*p*'< 0.0001, 'p' value ***).

Further, the mRNA expression of TIMP genes (TIMP-1/ TIMP-3) was elucidated in the ECT of *C. trachomatis*-positive spontaneous aborters by using RT- PCR. Housekeeping gene β-actin was used as an internal control. In all *C. trachomatis*-positive endometrial tissue specimens, signals were detected for the cDNA amplification products of the TIMP-1/ TIMP-3 **(Fig. 6.3 a, b).** The expression of TIMP-1 and TIMP-3 was significantly low in *C. trachomatis*-positive spontaneous aborters in comparison to the control group ($p$<0.0001; $p$<0.01 arbitrary units respectively; **Fig. 6.4 a, b).**

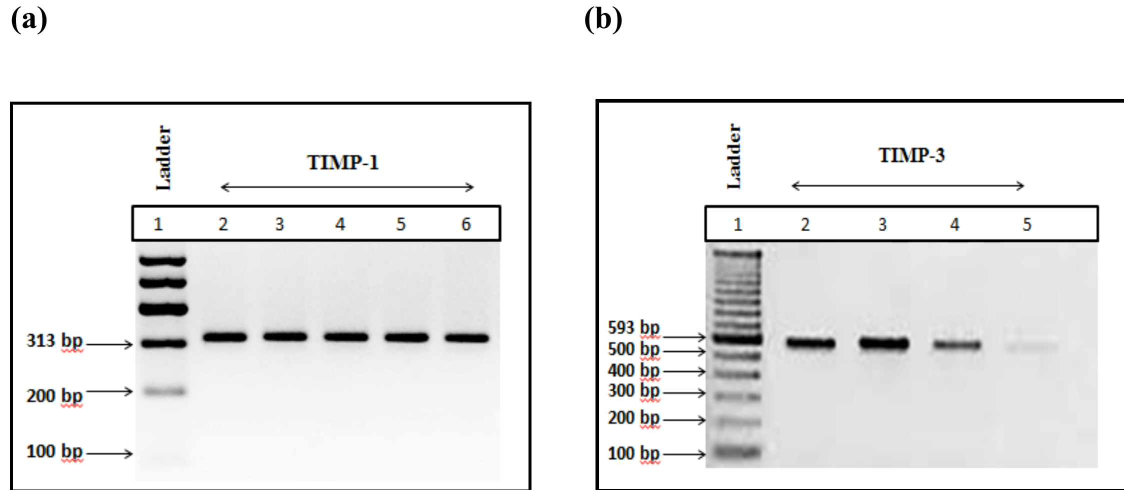



**Fig. 6.3 a, b:** Amplification products for TIMPs in ECT of spontaneous aborters. (a) is showing the amplification products of TIMP-1 (b) shows the amplification products of TIMP-3; Lane- 1shows 100 bp ladder, lanes 2 - 6 show TIMP-1 and TIMP-3 expression in the ECT of *Chlamydia trachomatis*-positive spontaneous aborters

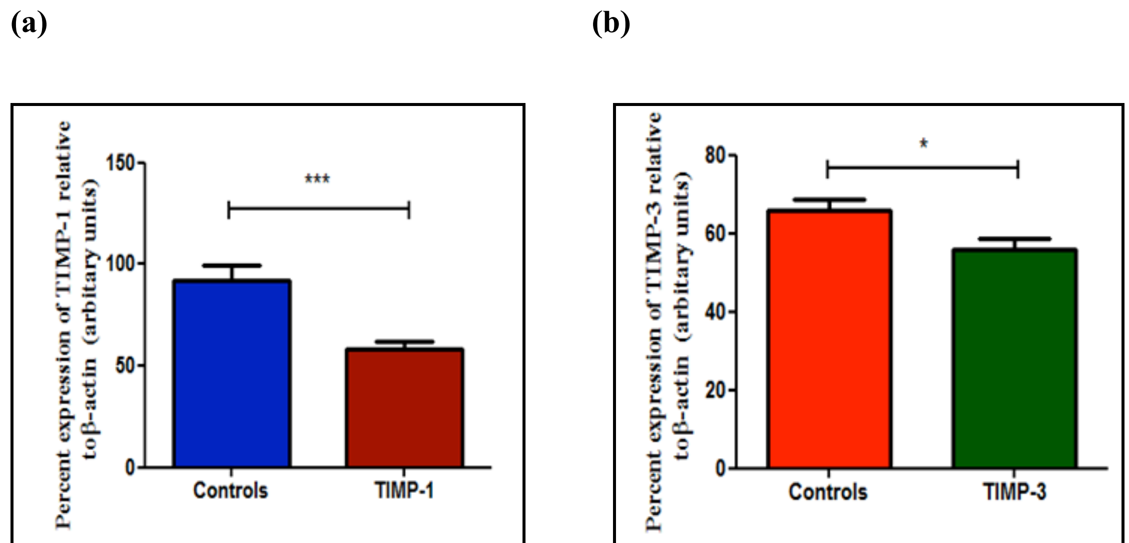



**Fig 6.4 a, b:** Expression of TIMP-1 and TIMP-3 genes in *Chlamydia trachomatis*-positive spontaneous aborters (n=22) *versus* control group by RT-PCR (Non-parametric Mann-Whitney test ‘*p*’< 0.0001) (‘p’ value *** = < 0.0001; * = < 0.05).

### 6.2.2. Quantitative analysis of mRNA expression for MMP-2/ MMP-9 and TIMP-1/ TIMP-3 genes by real time PCR

The expression of MMP-2 and MMP-9 genes was studied in the ECT at transcript level by SYBR green q-PCR and the expression was compared among patient group as a ratio to the expression of the constitutively expressed housekeeping β-actin gene in *C. trachomatis*-positive SA. Analysis of Ct values showed that the mRNA expression of MMP-2/ MMP-9 was significantly increased in comparison to controls. In comparison to *C. trachomatis*-negative SA, the expression of MMP-2/ MMP-9 was found significantly high in *C. trachomatis*-positive SA. (non-parametric Mann-Whitney test; '$p$' < 0.0001) **(Fig. 6.5 a, b).** However MMP-2 and MMP-9 expression in endometrial tissues harvested from women suffering from incomplete spontaneous abortions compared to ECT from control women was 3.1 and 5 fold higher, respectively.

Analysis of the gene expression of tissue inhibitors of matrix metalloproteinases TIMP-1/ TIMP-3 showed that the expression was significantly low in *C. trachomatis*-positive SA and in *C. trachomatis*-negative SA *versus* controls (non-parametric Mann-Whitney test; '$p$' < 0.0001) **(Fig. 6.6 a, b).**

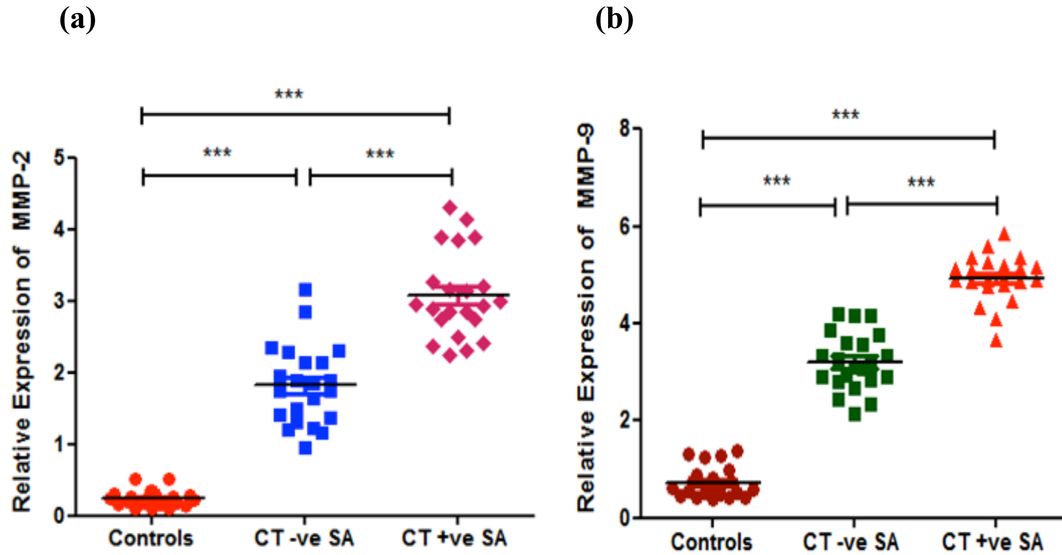



**Fig. 6.5 a, b:** Expression of MMP-2 and MMP-9 gene was significantly high in *C. trachomatis* positive spontaneous aborters (Group I) (Non-parametric Mann-Whitney test; ***'*p*'< 0.0001).

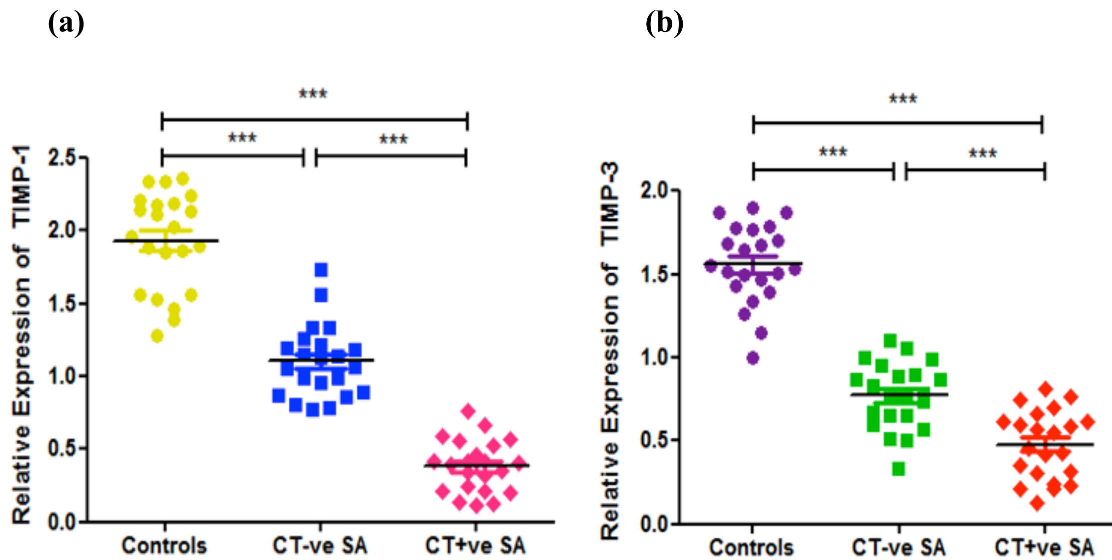



**Fig. 6.6 a, b:** Expression of TIMP-1 and TIMP-3 gene was significantly low in *C. trachomatis* positive spontaneous aborters (Group I) versus controls (Non-parametric Mann-Whitney test; ***'*p*'< 0.0001) ('*p*' value *** = < 0.0001).

**Table 6.1:** Comparison of relative expression of MMP-2/ MMP-9 and TIMP-1/ TIMP-3 mRNA in the endometrial curettage tissue of the study and control group. Values presented are the Mean values.

| | | | |
|---|---|---|---|
| | **MMP-2** | **TIMP-1** | **MMP-2/ TIMP-1** |
| | 3.1 | 0.3 | 10 |
| ***C. trachomatis* +ve SA** | | | |
| **(n = 22)** | **MMP-9** | **TIMP-3** | **MMP-9/ TIMP-3** |
| | 5.0 | 0.46 | 12.5 |

### 6.2.3. Correlation between TNF-α, MMP-2 and MMP-9 expression

Correlation was calculated between the TNF-α and MMP-2/ MMP-9 expression in the ECT by using spearman rank correlation test. A statistically significant positive correlation was observed between TNF-α and MMP-2 expression in the ECT of *C. trachomatis*- positive women (r = 0.70; '*p*' = 0.0009, **Fig. 6.7**). However, the expression of MMP-9 in the ECT was not significantly correlated with the TNF-α hence in the study group we have not been able to show any correlation between expression of TNF-α and MMP-9 (r = 0.08, '*p*'= 0.71; not-significant).

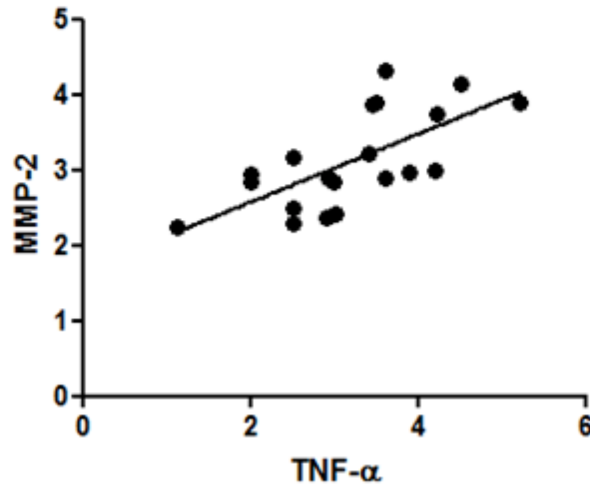



**Fig. 6.7:** Determination of correlation between MMP-2 and TNF-α with spearman rank correlation test (‘*p*’ < 0.05).

**6.2.4. Correlation between TGF-β1, MMP-2 and MMP-9 expression**

A correlation was also made between the mRNA levels of MMP-2 and TGFβ1. There was a statistically significant positive correlation was observed between TGF-β1 and MMP-2 expression in the endometrial tissue of *C. trachomatis*- positive women (r = 0.80; ‘*p’* < 0.0001) **(Fig. 6.8),** which means, that a higher MMP-2 expression was accompanied by a higher TGF-β1 expression. However, the expression of MMP-9 in the ECT was not significantly correlated with the TGF-β1 hence in the study group we have not been able to show any correlation between expression of TGF-β1 and MMP-9 (r = 0.18, ‘*p’*=0.44; not-significant).

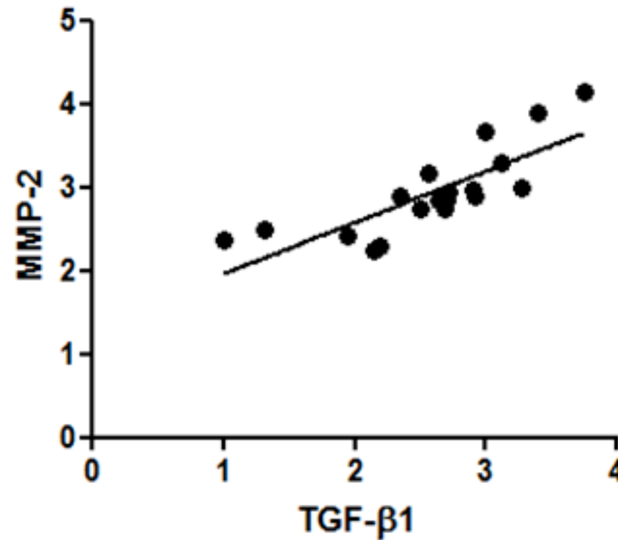



**Fig. 6.8:** Determination of correlation between MMP-2 and TGF-β1 with Spearman rank correlation test: MMP-2 and TGF-β1 correlation (‘*p*’ < 0.0001).

### 6.2.5. Correlation between TGF-β2, MMP-2 and MMP9 expression

A statistically significant positive correlation was also observed between TGF-β2 and MMP-2 expression in the endometrial tissue of *C. trachomatis*- positive women (r = 0.80; ‘*p’* < 0.0001) **(Fig. 6.9),** which means that the higher MMP-2 expression was accompanied by a higher TGF-β2 expression. However, the expression of MMP-9 in the ECT was not significantly correlated with the TGF-β2 in the study group (r = 0.16, ‘*p’*=0.42; not-significant).

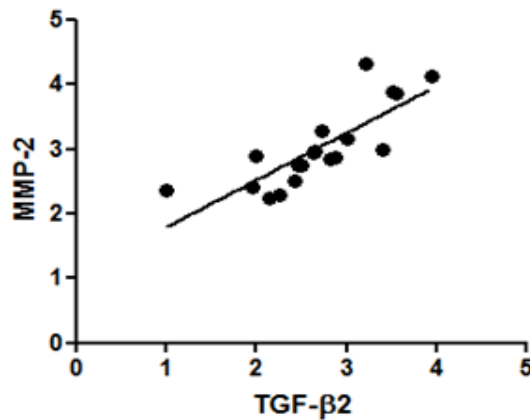



**Fig. 6.9:** Determination of correlation between MMP-2 and TGF-β2 with Spearman rank correlation test: MMP-2 and TGF-β2 correlation (‘$p$’ < 0.05)

### 6.2.6. Correlation between MMP-2, MMP-9 and COX-2 expression

The MMP-2/ MMP-9 genes were further correlated with Cox-2 gene expression in the ECT. It was found that MMP-2 mRNA level was correlated positively with the mRNA levels of COX-2 (r = 0.70; ‘$p$’ = 0.001, **Fig. 6.10**) while in the study group we have not been able to show any correlation between the expression of MMP-9 and COX-2 (r = 0.03, ‘$p$’= not-significant).

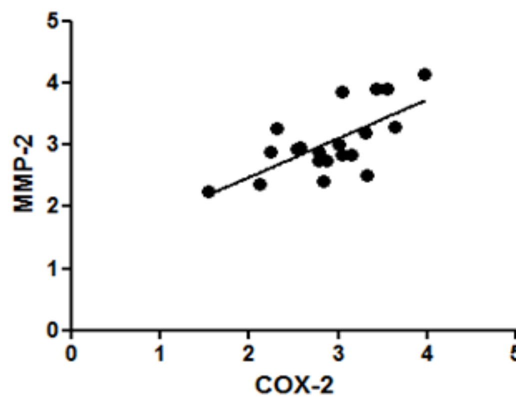



**Fig. 6.10:** Determination of correlation between MMP-2 and COX-2 with Spearman rank correlation test: COX-2 and TNF-α correlation (‘$p$’ significant).

### 6.2.7. Discussion

The maintenance of normal pregnancy is a synergic process between the embryo and the mother. Cytotrophoblast cells in normal early pregnancy secrete a large amount of MMPs and simultaneously generating TIMPs to inhibit MMPs, thus maintaining the dynamic balance during normal pregnancy **(Li *et al.,* 2006).** The majority of studies have shown that MMP-9 and TIMP-3 play particularly important roles in early pregnancy; the MMP-9/ TIMP-3 balance is significant in regulating the depth of invasion of cytotrophoblast cells into the uterus **(Walter & Schonkypl, 2006).** There have also been certain studies indicating that the high expression levels of TIMP-3 during endometrial implantation window may avoid the excessive degradation of ECM by MMP-9, prevent the infiltration of cytotrophoblast cells. There have been an increasing number of studies concerning the roles of MMPs and TIMPs in the abortion process, some of which have confirmed that in the MMP family. An imbalance in the expression of MMP-9 and TIMP-3 would affect fetal growth and development, and excessive invasion by cytotrophoblast cells is likely to result in miscarriage **(Li *et al.,* 2006).** It has been found that the MMP-9 activities in the endometria of women with failed early pregnancy are significantly increased and result in disorders of ECM components this suggests that MMP-9 is significant in the failure of pregnancy.

In the present study, the real time PCR was used to detect the expression of MMP-2/ MMP-9 and TIMP-1/ TIMP-3 mRNA in the endometrial tissues of SA patients, with the aim of exploring their correlation with *C. trachomatis*-induced spontaneous abortions. Our experiments suggest that MMP-2 and MMP-9 mRNA expression levels in the SA patients were significantly higher than those of controls and *C. trachomatis*-

negative SA (*'p'* <0.0001), suggesting that MMP-2/ MMP-9 plays an important role in infected SA. Table 6.1 demonstrates that the MMP-2/ MMP-9 expression level of the SA was significantly increased, indicating that the endometrium of the SA produced excessive amounts of MMP-2 and MMP-9. Our results are in accordance with a study reported that a higher expression of MMP-2 mRNA in the endometrium of women with recurrent miscarriage **(Jokimaa *et al.*, 2002).**

In the TIMP family of proteins, TIMP-3 has been shown to be the main inhibitor of MMP-9. In TIMP-3 enriched ECM, the degradation of ECM by growing blastocysts was also reduced compared with normal ECM; this decline was due to the inhibition of MMP-9 activity by TIMP-3. The present study detected the expression of TIMP-3 mRNA in the ECT of controls, and the results revealed that the TIMP-3 mRNA expression levels in the *C. trachomatis*- positive SA was low in comparison to the controls (*'p'* >0.0001).

In the present study, the ratio of MMP-2/TIMP-1 and MMP-9/TIMP-3 mRNA expression in *C. trachomatis*- positive SA was significantly higher than that in the control group (*'p'* <0.0001). From these results, it may be speculated that the imbalance in the levels of MMP-2/ MMP-9 and TIMP-1/ TIMP-3 is likely to cause excessive degradation of the ECM, thus affecting embryo implantation and even resulting in pathological spontaneous abortion. Because an increase in the MMP/TIMP ratio promotes matrix degradation, a 10 times higher ratio of MMP-2/TIMP-1 and 12 times higher ratio of MMP-9/TIMP-1 in *C. trachomatis*-positive spontaneous aborters suggests excessive degradation of the endometrial matrix which, in turn, affects proper remodelling of the

endometrium. A 25 times higher ratio of MMP-9/TIMP-1 was also seen in women suffering with IRSM **(Banerjee *et al.*, 2013).**

Further, TGFβs modulate maternal immunotolerance during implantation and regulate *in-vitro* several molecules related to implantation as MMP-9 **(Dimitriadis *et al.*, 2005).**TGF-β1 inhibits placental differentiation and invasion and extracellular matrix deposition and anti-inflammatory effects of TGF-β1 during pregnancy have been suggested by *in vitro* findings **(Goodwin *et al.*, 1998).** Also in the endometria from women with unexplained recurrent miscarriages, a higher TGF-β2 expression was noticed and the study suggested that pregnancy loss occurred due to the dysregulated TGF-β2, MMP-2, MMP-9 and TIMP-1 **(Skrzypczak *et al.*, 2007).** In our study, TGF-β1 and TGF-β2 were found positively correlated with MMP-2 in *C. trachomatis*-positive SA ('*p*' < 0.05), which means, that a higher MMP-2 expression was accompanied by a higher TGF-β1 expression. However, the expression of MMP-9 in the ECT was not significantly correlated with the TNF-α/ TGF-β1/ TGF-β2. Also, COX-2 is known to upregulate the expression of MMP-2 and MMP-9 **(Itatsu *et al.*, 2009)**. These enzymes are the main endometrial remodelling factors for implantation, and their activities depend on the balance of their inhibitors. We observed a statistically significant positive correlation between the COX-2 and MMP-2 expression in the ECT of *C. trachomatis*-positive SA.

In summary, the MMP-2 and MMP-9 mRNA expression levels of the infected patients undergoing SA were high compared with those in the controls, and the MMP-2/TIMP-1 and MMP-9/TIMP-3 mRNA ratio was also high in the SA. The results of this study provide an experimental basis for the clinical investigation of the mechanism of abortion, and also a theoretical basis for the clinical application of MMP-2

and MMP-9 as a diagnostic indicator of spontaneous abortion. In addition, the study supports the use of synthetic TIMPs to treat spontaneous abortion and maintain a normal pregnancy.

# CONCLUSIONS & FUTURE SCOPE OF WORK

# *Conclusions*

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In the present study, cyclooxygenase (COX)-derived prostaglandin (PG) signalling pathway was studied in *Chlamydia trachomatis*-infected spontaneous aborters (SA) to explore a new potential mechanism for spontaneous abortion in infected women. Since the exact pathway leading to infection-induced abortion has not yet studied, the present study has provided an improved understanding of involvement of varied immunomolecular aspects during spontaneous abortion in *C. trachomatis*-positive women.

1. The first aim of the study revealed that the expression of COX-1 in the endometrial curettage tissue (ECT) was neither significant in the *C. trachomatis*-positive SA nor in *C. trachomatis*-negative SA in comparison to the uninfected controls and COX-1 expression did not change with the SA; while the expression of COX-2 increased significantly in *C. trachomatis*-positive SA and also in uninfected SA in comparison to age-matched control women. Increased expression of COX-2 in infected SA was attributed to chlamydial infection. PGE2 contractile receptors/ relaxant receptors, PGF2α contractile receptor FP and PGI2 relaxant receptor, IP were significantly increased in *C. trachomatis*-positive SA *versus* controls/ *C. trachomatis*-negative SA. The expression of PG receptors was found increased significantly with the increasing GA. Also, the contractile EP-1/ EP-3/ FP receptors were positively correlated while the relaxant EP-2 and EP-4 receptors were found negatively correlated with GA in *C. trachomatis*-infected SA. Mean serum estrogen concentration was significantly high in *C. trachomatis*-positive SA, as compared

to both uninfected SA and the controls group; while, progesterone concentration was found to be low in *C. trachomatis*-positive SA. Our findings delineate that *C. trachomatis* infection might have led to an increase in COX-2 level in SA ultimately leading to an increased PG synthesis within the foetal membranes. Increased expression of COX-2 and PG receptors with advanced GA and imbalance in hormonal levels could be a possible risk factor for abortion in *C. trachomatis*-infected SA.

2. The second aim of the study revealed the modulatory effect of cytokines on *C. trachomatis*-positive SA. Significantly increased expression of proinflammatory cytokines (*viz.*: TNF-α, IFN-γ, IL-8) was found in the ECT of *C. trachomatis*-positive SA in comparison to controls. TNF-α, IFN-γ and IL-8 genes expression was significantly higher in the recurrent spontaneous aborters (RSA) in comparison to sporadic spontaneous aborters (SSA). The expression of multifunctional cytokines TGF-β1/ TGF-β2 was also found significantly enhanced in *C. trachomatis*-positive SA in comparison to controls. No significant difference was found in the expression of TGF-β1 and TGF-β2 between *C. trachomatis*-positive SA versus *C. trachomatis*-negative SA. Also, TNF-α/ TGF-β1/ TGF-β2 and COX-2 were found positively correlated with each other in *C. trachomatis*-infected SA. Results suggest that an aberration in cytokine production also constitutes an important contributing factor in *C. trachomatis*-positive SA and suggested involvement of these cytokines in the immunopathogenesis of SA. Also, cytokines specially TNF-α and IFN-γ can induce COX-2 expression and cause increased PG synthesis leading to spontaneous endometrial contractions and relaxations ultimately leading to SA.

3. Third aim of the study showed the involvement of matrix metalloproteinases (MMP-2/ MMP-9) and their inhibitors (TIMP-1/ TIMP-3) in endometrial extracellular matrix (ECM) turnover in *C. trachomatis*-infected SA. Expression of MMP-2/ MMP-9 was found significantly high in the ECT of *C. trachomatis*-positive SA in comparison to the *C. trachomatis*-negative SA and controls. TIMP-1/ TIMP-3 were significantly low in *C. trachomatis*-positive SA and in *C. trachomatis*-negative SA *versus* controls. The expression of MMP-2 was found positively correlated with TNF-α/ TGF-β1/ TGF-β2/ COX-2 in *C. trachomatis*- positive women. It was concluded that dysregulated MMPs/ TIMPs could degrade the endometrial ECM leading to spontaneous abortion in *C. trachomatis*-infected women. COX-2 and cytokines (*viz.*: TNF-α/ TGF-β1/ TGF-β2) in turn upregulated MMP-2/ MMP-9, leading to excessive endometrial matrix degradation, a possible cause of spontaneous abortion.

On an experimental basis, the results of entire study provide an immunomolecular mechanism **(Fig. 7)** leading to spontaneous abortion in *C. trachomatis*-infected women. Study also provides a theoretical basis for the clinical investigation of COX-2/ MMP-2 and MMP-9 as diagnostic indicators of spontaneous abortion for management of early pregnancy loss after validation in larger number of SA.

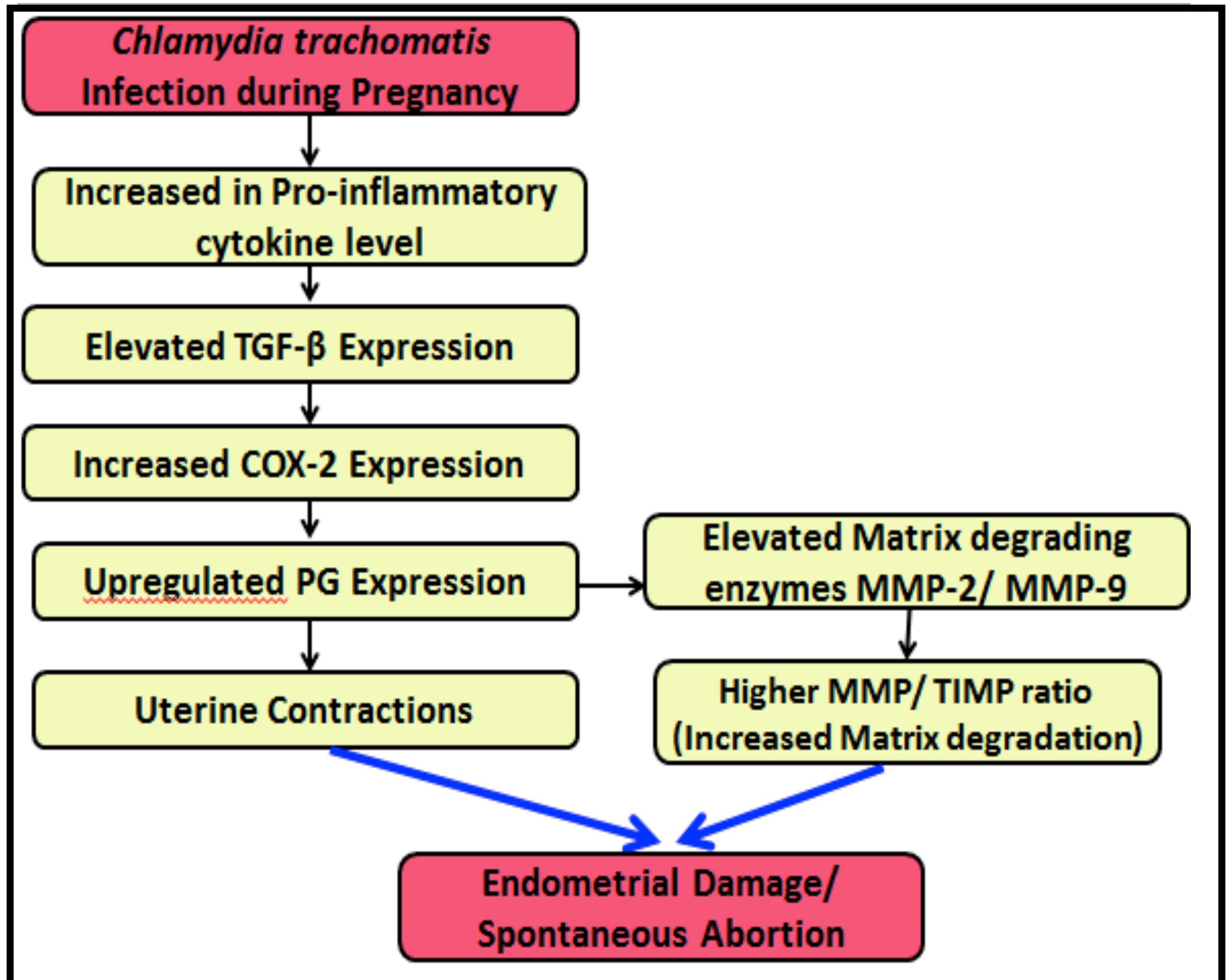


**Fig. 7:** Schematic representation of immunomolecular pathway leading to spontaneous abortion in *C. trachomatis*-infected women.

## *Future Scope of Research*

The present study is of therapeutic importance and has paved the way for better management of spontaneous abortion in women found infected with *Chlamydia trachomatis* and suffering recurrent miscarriage. However, there are certain limitations and validation studies are clearly warranted in larger number of such patients. Possibly, multicentric studies could be undertaken in a varied population of spontaneous aborters with confounding factors only; this should help in developing suitable biomarkers/ identifying suitable interventions for better management of pregnancy in clinical settings in India, where frequency of *C. trachomatis* has been reported to be high in ante-natal women (21%) and in spontaneous aborters (15%).

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## *List of Publications*

1. **Singh N**, Prasad P, Kumar P, Singh LC, Das B, Rastogi S (2016). Does aberrant expression of cyclooxygenase-2 and prostaglandin-E2 receptor genes lead to abortion in *Chlamydia trachomatis*-infected women. ***The Journal of Maternal-Fetal & Neonatal Medicine, 29:1010-1015***
2. **Singh N**, Prasad P, Singh LC, Das B, Rastogi S (2016). Expression of prostaglandin receptor in *Chlamydia trachomatis*-infected recurrent spontaneous aborters. ***Journal of Medical Microbiology**. **65:476- 483***
3. **Singh N**, Prasad P, Das B, Rastogi S (2017). Involvement of matrix metalloproteinases and their inhibitors in endometrial extracellular matrix turnover in *Chlamydia trachomatis*-infected recurrent spontaneous aborters. ***Pathogens and Disease (doi: 10.1093/femspd/ftx007)***
4. Prasad P, **Singh N**, Das B, Raisuddin S, Dudeja M, Rastogi S (2017). Circulating Th1/ Th2/ Th17 cytokines in the serum of *Chlamydia trachomatis*-infected first trimester spontaneous aborters: A new paradigm. ***Microbial Pathogenesis (doi: 10.1016/j.micpath.2017.06.031)***

**Publications in Proceedings:**

1. **N. Singh**, P. Prasad, B. Das, S. Rastogi (2016). Recurrent Spontaneous abortion: Significance of early non-invasive detection of *Chlamydia trachomatis* infection. ***International Journal of Infectious Diseases 45, (Suppl.1): P 47***

2. P. Prasad, **N. Singh**, B. Das, S. Raisuddin, M. Dudeja, S. Rastogi (2016). Differential expression of superoxide dismutases in early aborters infected with *Chlamydia trachomatis*. ***International Journal of Infectious Diseases 45, (Suppl.1): P 203-4***

**Presentations:**

1. Oral presentation "Recurrent spontaneous abortion: Significance of early non-invasive detection of *Chlamydia trachomatis* infection" presented **at 17th International congress on Infectious Diseases (ICID)**, held at Hyderabad, India- March 2016.

2. Oral presentation "Association of *Chlamydia trachomatis* with mRNA expression of proinflammatory cytokines and cox-2 genes in spontaneous aborters" presented at 18th **International Union against Sexually Transmitted Infections (IUSTI)**, Asia Pacific Conference in **Bangkok, Thailand** - November 2014.

3. Poster presentation "Role of Tumor Necrosis Factor alpha and Cyclooxygenases in women undergoing *Chlamydia trachomatis* infected spontaneous abortion". **Namita Singh**, Dr. Banashree Das, Dr. Sangita Rastogi at **IMMUNOCON**, November 2013.

## *Brief Biography of Candidate*

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**Ms. Namita Singh, M.Sc. Biotechnology**

I received my Bachelor degree (B.Sc. Honors Zoology) from Sri Venkateswara College, University of Delhi. After that, I pursued M.Sc. in Biotechnology from Banasthali University, Rajasthan. In masters, I received an award of scholarship from Department of Biotechnology, Government of India. During M.Sc., I did one month summer training from Institute of Cytology and Preventive Oncology (ICMR), Noida, India on “Basic Molecular Biology Techniques used in Cancer Research”. In the final year of my M.Sc., I also undergone six months project dissertation at Indian Institute of Technology-Delhi under the advisement of Prof. Saroj Mishra on the topic titled “Expression & Regulation of Laccase in *Cyathus bulleri*” and learnt various molecular biology and culture techniques as well as biochemical methods.

I qualified CSIR-UGC NET-JRF (National Eligibility Test**)** in December 2008 and Graduate Aptitude Test for Engineering (GATE) Examination in 2009. Then I joined National Institute of Pathology (ICMR), New Delhi as a UGC-JRF and started working here under the supervision of Dr. Sangita Rastogi, Scientist-F on *Chlamydia trachomatis*-induced spontaneous abortion. In 2011, I was awarded as UGC-senior research fellow. During the tenure at the institute, I got registered at Dept. of Biological Sciences, BITS-PILANI for pursuing Ph.D. and have been able to publish 4 research papers (3-original articles and 2-abstracts) from my thesis in peer-reviewed national and international journals. I had an opportunity to present my research work as two oral presentations and one poster presentation at international/ national conferences. I attended hands-on workshops on various aspects like proteomics techniques, application of statistical software in medical research, sequencing experimental design and quantitative genomics, Flow-cytometry and scientific writing. I was awarded as “Young investigator from India & South-east Asia” by Bill and Melinda Gates Foundation at International congress on Infectious Diseases (ICID), held at Hyderabad and also received travel grant award for an Oral Presentation by Indian Council of Medical Research, Government of India for my oral presentation at IUSTI-2014, Bangkok, Thailand.

## *Brief Biography of Supervisor*

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**Dr. Sangita Rastogi, M.Sc., M.Phil., Ph.D. (Zoology)**

Dr. Sangita Rastogi has been the recipient of '*Young Scientists Gold Medal'* by Society of Biosciences and was awarded direct SRF (1990-93) by CSIR during her Ph.D. (1987-92). Subsequently, she was awarded direct RA (1993) by CSIR for conducting research on endocrinal regulation of hepatocellular toxicity. She ultimately joined National Institute of Pathology (NIP), ICMR, New Delhi as Senior Research Officer/ Scientist 'C' in December 1993 and is presently working as Scientist 'F' & Senior Deputy Director. Dr. Rastogi heads the Microbiology Division at the Institute and her laboratory focuses on immunology of chlamydial infections. Besides this, she is also Officer-InCharge, Central Animal House Facility and Library at NIP. For the past 23 years onwards, she is engaged in chlamydial research on cellular/ molecular mechanisms of immunopathogenesis with an ultimate aim to develop biomarkers/ preventive interventions for patients suffering from reactive arthritis, adverse obstetric outcomes and infertility. She is also member of various international/ national scientific organizations, *viz.:* ISID, ARHP, IIS and STOX. She is teaching faculty for WHO-sponsored training program for pathologists/ technicians, Off-campus Pre-Ph.D. program of BITS (Pilani), Symbiosis International University (Pune), GGSIP University (New Delhi) and Jamia Hamdard University (New Delhi) at NIP. She has supervised several thesis of post-graduate (M.Sc.), Ph.D., M.S. (Obstetrics & Gynecology) students. Dr. Rastogi has 37 research papers (*h*-index: 9) in peer-reviewed journals to her credit and has also contributed one chapter in book. Two of her research publications in *Chlamydia trachomatis*-induced reactive arthritis have been cited in 2016 as articles of major importance by renowned American and German rheumatologists. She has handled/ supervised extramural research projects funded by LSRB (DRDO), DOE (MIT), DST and ICMR in varying capacities as PI/ Co-PI/ CoI. She has actively participated and presented her research in various national/ international conferences in both India and abroad. She is the recipient of international travel grant awards by ICMR, CICS and DST for participation and presentations in various scientific conferences.

## *Brief Biography of Co-supervisor*

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**Professor Vishal Saxena, M.E., Ph.D.**

Prof. Vishal Saxena is working as an Associate Professor at Molecular Parasitology & Systems Biology Lab, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan, India. His major thrust area of research is Molecular Biology and Immunology with special emphasis to Genomics and Proteomics of malaria parasites; *Plasmodium vivax* and *P. falciparum*. His group is focusing on various aspects related to *P. vivax* infections in humans, apicoplast and its genome, metabolic pathways functional in the apicoplast, hypothetical proteins encoded by *P. vivax* nuclear genome. He was a Visiting Scholar at Department of Public Health, College of Global Health, University of South Florida, Tampa, Florida, USA from June - July 2015. Prof. Saxena obtained his bachelor degree in Pharmacy (University of Rajasthan, Jaipur, August 1999) followed by M. E. Biotechnology (BITS- Pilani, June, 2001) and Ph. D. (Biological Sciences Group, BITS, Pilani, October, 2006). He has a total 16 years (5 years Pre-doctoral experience, over 11 year job tenure) of research experience. He is actively involved in teaching and research and has handled projects from various funding agencies, has supervised many graduate and post-graduate students thesis and is currently supervising 5 Ph.D. students. He was a recipient of Young Scientist Award for Best Poster Presentation at International Conference on Molecular Epidemiology & Immunology of Malaria and other Vector Borne Diseases at RMRCT, Jabalpur, M.P., INDIA, 2007. He has published more than 15 research papers in international journals and has authored a book on Genetic Engineering. He has participated in many national and international conferences in India and abroad too.